5-2017

TUMOR-CELL DERIVED INHIBIN, BETA A IS ESSENTIAL FOR ADRENERGIC EFFECTS ON FIBROBLAST ACTIVATION

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A

THESIS

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

May 2017

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Abstract

Objectives: Catecholamine-mediated effects driven by elevated adrenergic signaling are known to increase tumor growth and metastasis by direct effects on tumor cells. However, knowledge of effects of adrenergic signaling on other cells such as cancer-associated fibroblasts (CAFs) within the tumor microenvironment is limited. We hypothesize that adrenergic signaling and norepinephrine can accelerate the induction of CAF-phenotype to promote inflammation and metastasis.

Methods: Ingenuity Pathway Analysis and NetWalker were used to assess gene expression data from patients with known CESD (Center for Epidemiological Studies-Depression)-score alongside microdissected CAFs from primary ovarian cancer. Tumor samples from mice exposed to daily restraint-stress or non-stressed controls were assessed for alpha-smooth muscle actin (a-SMA) expression. Normal fibroblasts (NoF 151) were treated with conditioned media from non-treated and norepinephrine (NE) treated Skov3 and HeyA8 cells and analyzed for induction of CAF-phenotype by a-SMA expression. Gene and protein expression of cytokines and changes in migratory potential of fibroblasts were assessed.

Results: Among the significantly upregulated genes (fold-change >2) in patients with known CESD-score, 34 genes overlapped with microdissected CAF data. Among them are several networks involved in extracellular matrix (ECM) and inflammatory response. Restraint stress was associated with significantly increased levels of α -SMA, a marker for CAFs by both intensity and distribution in Skov3, HeyA8 and ID8 tumors. These increases in α-SMA were abrogated when mice were treated with broad beta-blocker propranolol during restraint stress in HeyA8 and Skov3 model. Further bioinformatics analysis of these datasets combined with gene array data from conditioned normal fibroblasts showed collagens are the most important biological downstream effectors for the adrenergic induced CAF phenotype. Consistent with the bioinformatics analyses, there was increased collagen (assessed by Trichrome stain) in the tumors from restraint-stress animals, and these were abrogated by propranolol treatment. Upstream bioinformatics analysis of CAF mediators showed cancer-cell derived INHBA (inhibin beta A, a member of the TGF-b family) can mediate the induction of CAF-phenotype upon adrenergic stimulation. Silencing INHBA in tumor cells decreased stress-induced tumor growth in Skov3 orthotropic model as well as decreasing levels of CAFs and collagen in tumors.

Conclusion: Sustained adrenergic stimulation results in significant increases in the CAF content *in-vivo* and accelerates conversion of normal fibroblasts to CAFs *in vitro* (fold change-2 fold, $p<0.05$). Adrenergic-mediated changes in CAF phenotype can increase production of pro-inflammatory cytokines and promote

inflammation. This project provides a better understanding of the adrenergic influences on the stroma within the tumor microenvironment.

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Hypothesis and Specific Aims

Hypothesis: Sustained adrenergic signaling accelerates CAF-phenotype & increases ECM matrix proteins primarily collagens in ovarian tumors.

Specific Aim 1: To determine the effects of chronic stress in promoting CAF phenotype in ovarian tumors.

Specific Aim 2: To identify the functional consequences of activation of CAFs during adrenergic signaling.

Specific Aim 3: To study the clinical relevance of stromal signature in ovarian cancer and applicability to other tumor models.

1 Introduction

1.1 Chronic Stress

1.1.1 History of the “General Adaptation Response”

A stress response is an evolutionarily conserved and non-specific adaptation mechanism that is triggered during distress or threats. Hans Selye pioneered the work on stress in 1930s [1, 2]. In his early studies, he noticed common symptoms such as loss-of appetite, muscle mass and anhedonia in patients who came to hospitals with very diverse ailments such as burns, end-stage cancer, infectious disease [2]. As a researcher who was studying the response of a new hormone in rats, Han Selye discovered what he defined as the triad of responses: enlarged adrenal glands, involuted lymphatic organs and severe bleeding in the duodenum and stomach [3]. He attributed this to the ‘General Adaptation Syndrome’ [4-6]. He further explained that this response consisted of 3 phases: alarm reaction, resistance and exhaustion. In the first phase, which is the alarm reaction, the body prepares to the threat by increasing levels of corticoids in the bloodstream and the adrenal glands are depleted of lipids. As this phase prolongs, the body enters the phase of resistance where there is an increased supply of corticoids and lipids in the adrenal glands accompanied by hemoconcentration and anabolism. The final stage, Hans Selye called the stage of exhaustion where the body is can no longer fight the severe stress [7]. The fundamental flaw with Selye’s experiments was that he hypothesized that stress hormones run out after chronic episodes of stress, but subsequent studies on stress responses showed that levels remain elevated and have several biological implications.

1.1.2 Stress Response

Chronic stress is defined as a nonspecific response of the body to any long standing demand. The ‘demands’ also known as stressors can be a physical or an emotional exertion, but the biological manifestation will be the same [8]. Colloquially called ‘the fight-or-flight’ response, this refers to an acute response that prepares the body to perceive and react to a threat. Such a response is both necessary and indispensable to animals. Acute stress responses are what enable a prey to run away form a predator, fight impending danger, and veer away from an incoming car [9]. These examples manifest acute stress, which is often short lived, transient and beneficial to the organism. In contrast, American Psychological Association defines chronic stress in individuals as a “cascade of negative emotions such as anxiety, anger and distress that also leads to predictable biochemical, physiological, and behavioral changes”[8].

1.1.3 Biology of the stress response

The normal biochemical manifestation of a stress response is the same irrespective of the source of stress. In response to a perceived threat, the hypothalamus is activated. There are 2 arms to the stress response: sympathomedullary pathway (SAM) or hypothalamic pituitary axis (HPA) that leads to production of norepinephrine (NE) and cortisol respectively (figure 1). In SAM, hypothalamic activation of the adrenal medulla results in the secretion of epinephrine. Adrenal medulla is part of the autonomic nervous system and chromaffin cells in the medulla are the primary source of catecholamines epinephrine and norepinephrine in the body. At times of high sympathetic nerve

activation, the amount of norepinephrine entering the blood increases dramatically. In HPA axis, hypothalamus activates the pituitary gland to produce adrenocorticotropic hormone (ACTH). ACTH then stimulates the adrenal cortex to produce corticosteroids such as cortisol that enters the bloodstream.

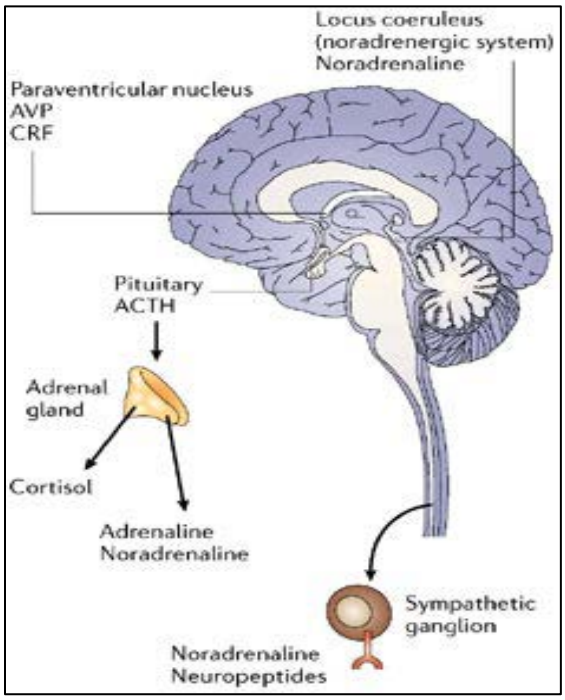

Figure 1: Stress Response. [10] (M.H. Antoni, S.K. Lutgendorf, S.W. Cole, F.S. Dhabhar, S.E. Sephton, P.G. McDonald, M. Stefanek, A.K. Sood, The influence of bio-behavioural factors on tumour biology: pathways and mechanisms, Nat Rev Cancer, 6 (2006) 240-248.) Nature Reviews Cancer by NATURE PUBLISHING GROUP. Reproduced with permission of NATURE PUBLISHING GROUP in the format Republish in a thesis/dissertation via Copyright Clearance Center.

In patients with cancer, both cortisol and norepinephrine levels are elevated with different biological functions. Lutgendorf et al studied NE levels in ovarian cancer

patients in both blood plasma and tumors [11]. In 68 ovarian cancer patients, tumor samples were collected and a pathologist confirmed for ovarian carcinoma histology were confirmed. Tumor samples were obtained from 20 patients, and plasma catecholamines were available for 53 patients (22 had ascites which is the fluid accumulation in the peritoneal cavity commonly seen in ovarian cancer patients). Patients were also assessed for biobehavioral profiles using CESD (Center for Epidemiological Studies-Depression) scores and assessed as high risk (score>=16) or low risk (score<15). Tumor samples and plasma were also analyzed for NE levels using HPLC (high performance liquid chromatography). Low risk patients showed minimal intra-tumor NE (all samples had less than <0.1 pg/mg tissue assay), whereas tissues from high-risk patients showed significantly greater NE concentrations with a significant increase in tumoral NE with a mean = 19.5 ± 6.9 pg/mg tissue (figure 2). There were no significant differences in plasma collected from the same patients 2 hours prior to resection surgery. The authors of the study also point that the differences between tumor and plasma levels of NE in the groups that catecholamines in peripheral circulation are likely not responsible for the bulk of the intratumoral catecholamine levels. Unpublished data in our lab has shown that chronic adrenergic signaling can lead to neoinnervation driven by BDNF produced by tumor cells. Peripheral nerves infiltrating the tumor can produce NE in the tumor, and the low acidic pH of the tumor microenvironment also helps in increasing the stability of NE within the tumor. Removal of the adrenal glands did not affect

tumoral NE levels, indicating that systemic sources of NE do not drive tumoral NE levels.

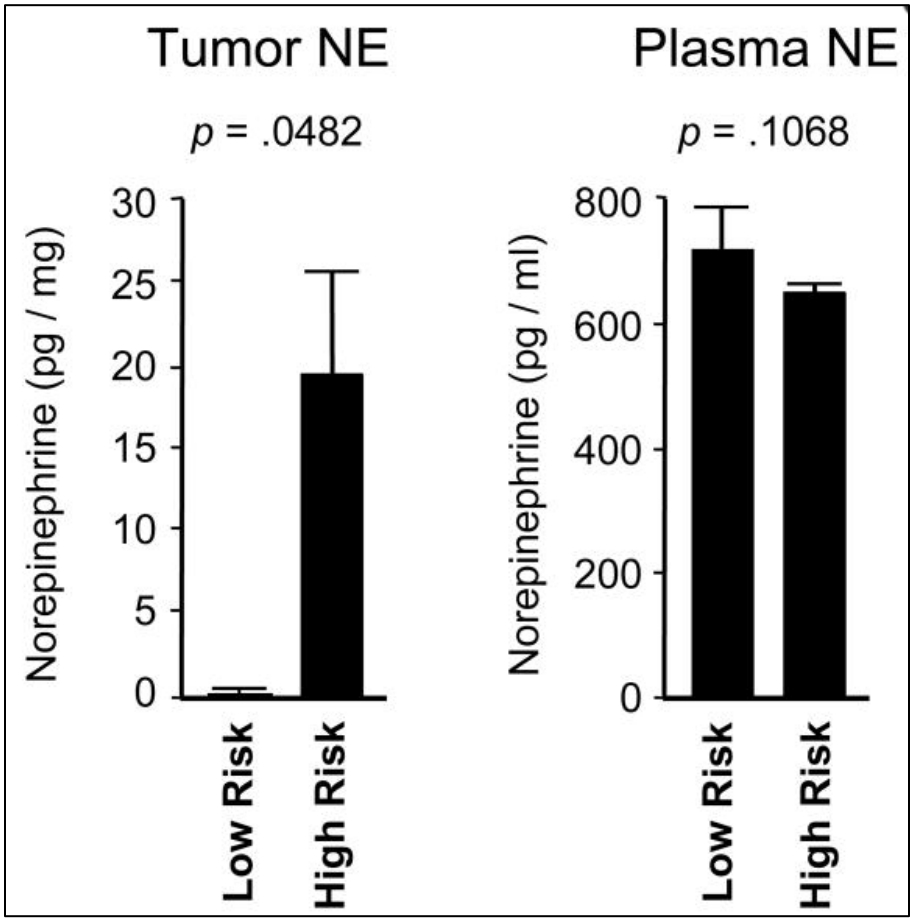

Figure 2: NE levels in ovarian tumors and circulating blood [11] (S.K. Lutgendorf, K. DeGeest, L. Dahmoush, D. Farley, F. Penedo, D. Bender, M. Goodheart, T.E. Buekers, L. Mendez, G. Krueger, L. Clevenger, D.M. Lubaroff, A.K. Sood, S.W. Cole, Social isolation is associated with elevated tumor norepinephrine in ovarian carcinoma patients, Brain Behav Immun, 25 (2011) 250-255.) Brain, behavior, and immunity by Psychoneuroimmunology Research Society Reproduced with permission of ACADEMIC PRESS in the format Thesis/Dissertation via Copyright Clearance Center. Order License 4081680368687

The primary reason to have a stress response was to be a protective response to a perceived or imminent threat. At the end of the stressful experience, the body gets back to normal by making biobehavioral or biochemical changes. This process is commonly referred to as allostasis [12]. Bruce McEwen in his seminal work on neurophysiology of stress, defined the term allostatic load as the impact on the body as it forced to adapt to adverse situation, either psychological or physical. He included that this can be attributed to either too much stress, or inability of the stress hormones to turn off after the stressor has passed.

He explained there are 4 types of allostatic loads commonly seen (figure 3). In a normal response, upon removal of stressor or after the perturbation has passed, the body enters a recovery state and returns to normal (top panel). In abnormal states, there could be a repeated hit or insufficient response. If a person is subject to repeated stressors over a long period of time, the levels of cortisol can stay elevated exacerbating the chronic effects of stress. In some individuals, there may be a lack of adaptation that prevents allostasis (middle panels). In some cases, there was an insufficient in the body's ability to produce appropriate responses. A prolonged response occurs when the signals indicated to stop the stress response are not triggered in a timely manner (bottom panel left). In general, catecholamines increase proinflammatory cytokines viaADRB2 receptor, whereas glucocorticoids inhibit production. An inadequate response is generated as shown (bottom panel right) when there is insufficient production of glucocorticoids during a stress response. This results in prolonged elevated

levels of proinflammatory cytokines (Nf-kappaB mediated cytokines) that cannot be sufficiently inhibited by low levels of glucocorticoids.

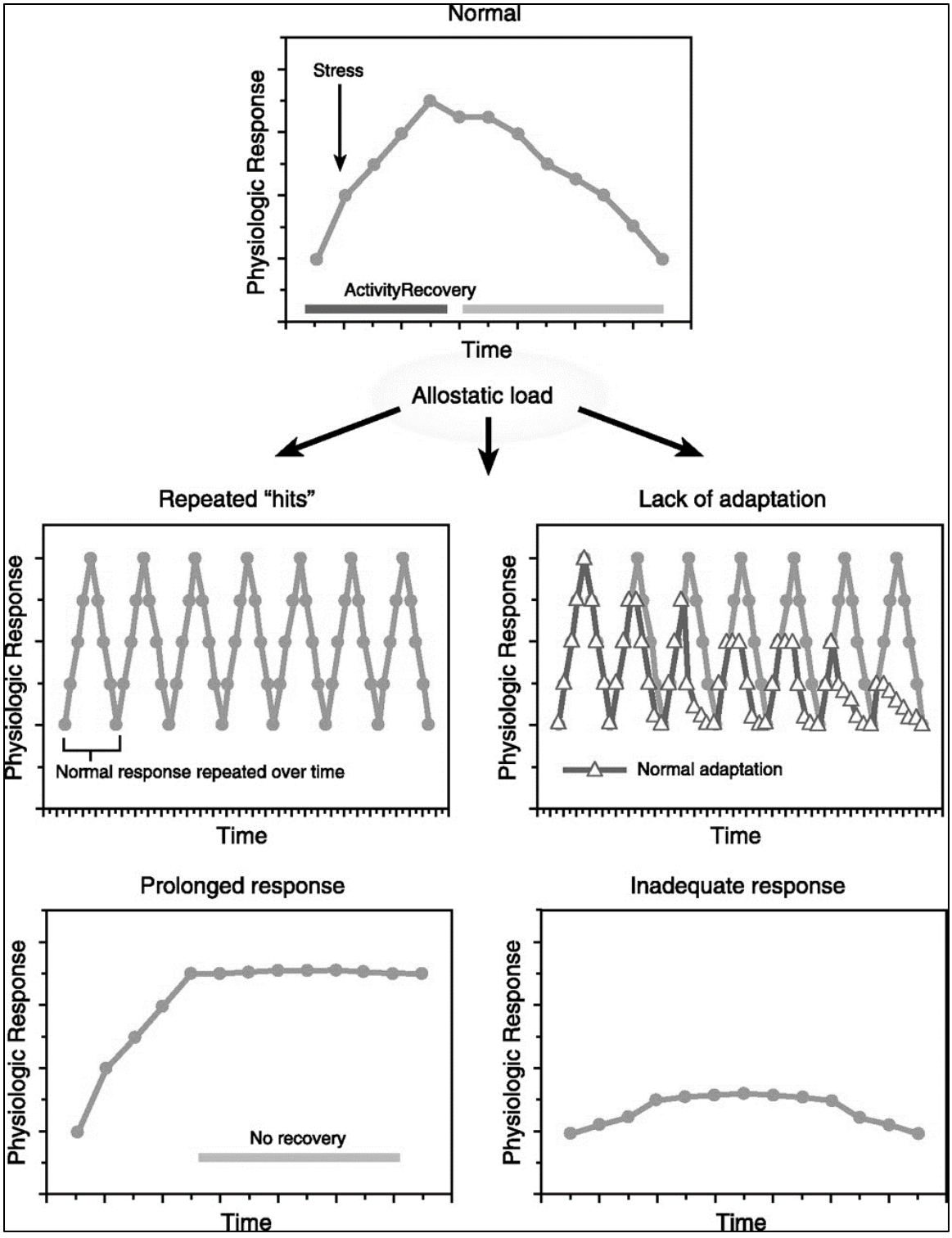

Figure 3: Allostatic load [13] (B.S. McEwen, Allostasis and allostatic load: implications for neuropsychopharmacology, Neuropsychopharmacology, 22 (2000) 108-124.) (Neuropsychopharmacology : official publication of the

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1.2 Acute v. chronic stress

In a typical stress response, as shown in figure 4**,** presence of a stressor is first accompanied by increased production of catecholamines within first 30 min [14, 15]. The catecholaminergic spike provides the rapid response needed to counter the stress by increasing via ADRB heart rate, alertness, reducing peripheral vision. The glucocorticoids spike an hour after the initial stressor. The role of glucocorticoids such as cortisol is to consolidate and terminate the stress reactions, mobilize energy resources required for this purpose and facilitate recovery. The long term genomic effect of GCs in the brain is to promote memory storage in preparation for future events. Catecholamines and GCs are the most important molecules produced in the stress response, and the following sections describe common signaling mechanisms and effector molecules attributed to both signaling pathways.

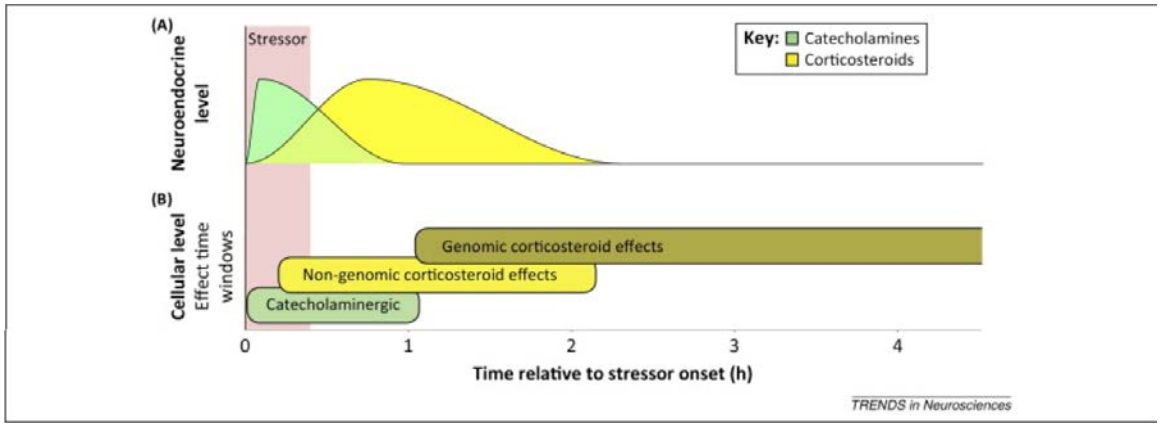

Figure 4: Stress Response and Time [14] (E.J. Hermans, M.J.A.G. Henckens, M. Joels, G. Fernandez, Dynamic adaptation of large-scale brain networks in response to acute stressors, Trends Neurosci, 37 (2014) 304-314.) Trends in neurosciences by ELSEVIER LTD.. Reproduced with permission of ELSEVIER LTD. in the format Thesis/Dissertation via Copyright Clearance Center. Order License Id: 4081680370430

1.2.1 Differences between catecholamines and corticosteroids

Catecholamines such as norepinephrine and corticosteroids such as cortisol have different functions during the typical stress response. Sympathetic nervous system is activated, accompanied by increased heart rate supplying blood to muscles and other organs, increased mobilization of energy, decreased digestion and increased breathing to maximize oxygen intake as the body prepares for the threat. Cortisol stimulates gluconeogenesis thereby providing a steady source of energy during the stress response. In addition, cortisol very potently decreases immune function by blocking release of inflammatory molecules and also greatly diminishes wound-healing capacity.

1.2.2 Adrenergic signaling

Catecholamine signaling in cells occurs via adrenergic receptors, which are all intronless 7-transmembrane G-protein coupled receptors. Epinephrine and norepinephrine signal via ADRA and ADRB receptors respectively. There are 5 alpha-adrenergic (ADRA) receptors ADRA1a, ADRA1b, ADRA2a, ADRA2b and ADRA2c, and 3 beta-adrenergic receptors (ADRB) ADRB1, ADRB2 and ADRB3. These receptors differ in the G-protein that it is associated with as well as their tissue distribution. G proteins are guanosine-binding messengers downstream of adrenergic receptors, and can be coupled to cAMP signaling (stimulatory (Gas), inhibitor (Gi)) or coupled to phospholipase C (Gq). Alpha adrenergic receptors are vasoconstrictive and play an important role in smooth muscle contraction ADRA1 associated with Gq subunit that is vital for PLC signaling and smooth muscle contraction. In heart, it activates a phosphatidylinositol-calcium second messenger system and is important for ERK-signaling. ADRA2 subtypes are coupled with Gi subunit that is the inhibitory and decreases both levels of cAMP and neurotransmitter release. This in turn decreased smooth muscle contraction.

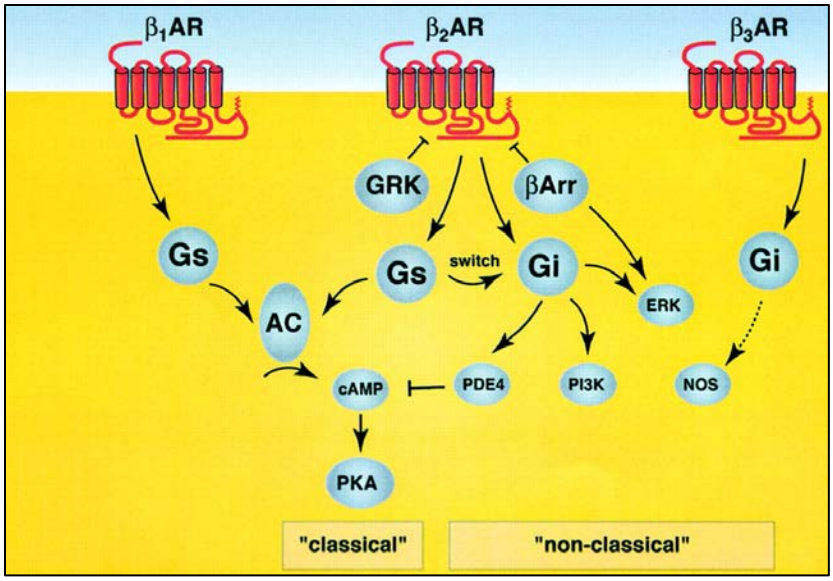

Figure 5: Beta-adrenergic signaling [16] (S.W. Cole, A.K. Sood, Molecular Pathways: Beta-Adrenergic Signaling in Cancer, Clin Cancer Res, 18 (2012) 1201-1206.) Clinical cancer research by American Association for Cancer Research ; HighWire Press Reproduced with permission of AMERICAN ASSOCIATION FOR CANCER RESEARCH in the format Thesis/Dissertation via Copyright Clearance Center. Order License ID: 4081680372059

ADRBs are all primarily coupled to Gs or stimulatory G-protein subunit (figure 5). Upon activation of receptor by norepinephrine, downstream signaling can activate several pathways in several discreet cell types, and due to their clinical importance, several agonists and antagonists for ADRBs have been identified and studied extensively (Table 1). ADRB1 is coupled to Gas, ADRB2 to both Gas and Gi and ADRB3 is coupled to Gi/nitric oxide pathway. Coupling to Gs increases levels of cAMP and binds the regulatory subunit of PKA and the activated catalytic subunit is released. PKA can phosphorylate serine and threonine proteins on downstream effectors that have the PKA-specific motif

(Arg-Arg-any amino acid-Ser/Thr-hydrophobic amino acid) [16]. One of the most important of this is phosphorylation of serine on CREB/ATF (cAMP Response Element Binding Protein/Activating Transcription Factor). Genome-wide transcription analysis identified 10,447 full CREs and 740,390 half CREs sites in the human genome with >4,000 sites on the genome where CREB proteins binds [17]. Some of the targets of the transcription factors include important genes related to transcription factor activation, cell metabolism, secretory factors and cell cycle. To a smaller extent, downstream activation can also activate Nf-kB, an important factor in promoting inflammation.

Type	**Potency (antagonist)**	**Tissue Distribution**	**Characteristics**
ß1	ISO > EPI = NE (Practolol, ICI 89,406)	Heart, lung, adrenal cortex, adipocyte	Cardiovascular signalling, lipolysis
ß2	ISO > EPI > NE (Butoxamine, ICI 118,551)	WBC, NK cells, lung, bone marrow, appendix	Bronchodilation, fight or flight response, glycogenolysis, insulin secretion
ß3	ISO > EPI (BRL-37344, Pindolol)	Salivary gland, omentum, thyroid, gall bladder	Lipolysis, thermogenesis

Table 1: Beta adrenergic receptor: functions, tissue distribution and chemical modulators

1.2.3 Glucocorticoid signaling

Cortisol is the active glucocorticoid that is formed by corticosterone by HSD11b1 (11β-Hydroxysteroid dehydrogenase type 1) enzyme (figure 6) [18, 19]. Glucocorticoid receptor (GR) is the nuclear receptor with three domains an N-terminal transactivation domain, a DNA-binding domain and a C-terminal ligand binding domain. The DNA-binding domain recognizes a DNA sequences called as GR- responsive element. GR is inactive and in cytoplasm bound to various factors but can be activated in the presence of cortisol. Glucocorticoids regulate gene expression in three different ways: the first is to bind directly to DNA the second one is to co-activate with other transcription factors bound to DNA and third is by alteration of kinases. While association of GR to members of STAT family of proteins enhance transcription, association of GR with Nf-KB decreased their activity. Glucocorticoids are well studied in their modulation of inflammatory pathways, specifically those related to Nf-kB. Reduced Nf-kB activity also results in decreased transcription of IL-1b and osteocalcin. Glucocorticoids have potent anti-inflammatory actions and exacerbate several diseases including osteoporosis, glaucoma, cardiovascular and psychiatric diseases. The same anti-inflammatory effects of GCs are also used to treat ailments driven by an inflammatory response such as rheumatoid arthritis, eczema, asthma and psoriasis [19-22]. Glucocorticoids were the first immunosuppressive drugs used in organ transplant patients due to their anti-inflammatory and immunosuppressive effects on several immune cells. GCs prevent dendritic cell

maturation, suppress migration of neutrophils and have moderate activity on both T and B cells [23-25].

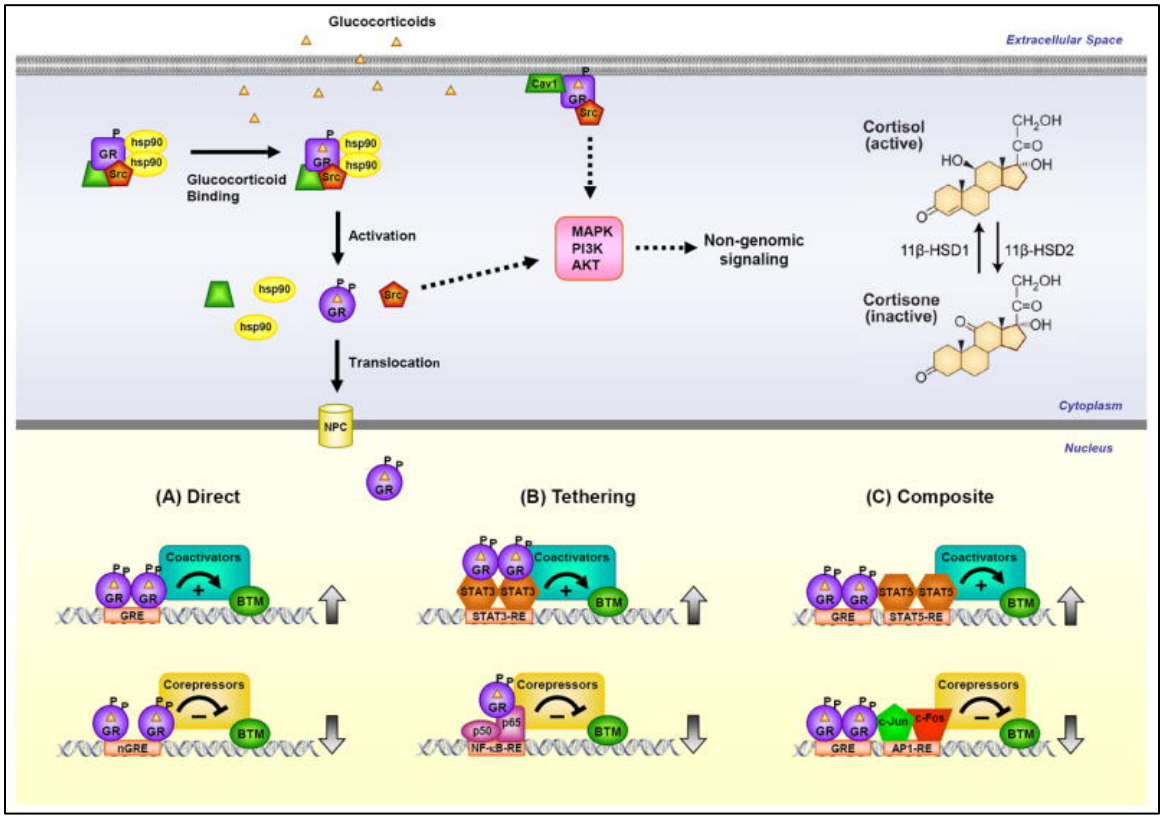

Figure 6: Glucocorticoid Signaling [18] (R.H. Oakley, J.A. Cidlowski, The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease, The Journal of allergy and clinical immunology, 132 (2013) 1033-1044.) The Journal of Allergy and Clinical Immunology : In Practice by Elsevier. Reproduced with permission of Elsevier in the format Thesis/Dissertation via Copyright Clearance Center.

1.3 Effects of chronic stress on disease

Chronic psychological stress manifests in different ways in different people. American Psychological Association defines stress as “uncomfortable emotional experience accompanied by a predictable biochemical, physiological or

behavioral changes" [26]. While acute stress is adaptive and can be beneficial and important from an evolutionary perspective to prepare for an imminent threat, chronic stress has no apparent benefits. Excessive chronic stress, which is constant and persists over an extended period of time either due to repeated stress or sudden events. Chronic stress can be physically and emotionally debilitating, and increase risks of disease. Longitudinal and retrospective studies studying the influence of psychological factors on human health and disease study several cohorts of people. Glaser et al, showed that individual's response for pathogens was significantly different between long term caregivers compared to non-caregivers due to altered immune response [27]. The same authors in another study showed that longstanding marital stress was associated with decreased immune function [28]. On the other hand, positive emotions and high social support was associated with increased NK function [29]. In a study involving 557 individuals, religious attendance was also associated with decreased IL6 levels and consequently played a role in better survival [30]. In SNS, hypersensitivity occurs during chronically stressful conditions. In preclinical models, animals exposed to chronic and repetitive stressors are able to produce and store higher amounts of catecholamines. These animals however do not show catecholamine surge to the same extent as control animals when exposed to the same stressor. However, presence of a new or novel stressor elicits an exaggerated response indicative of hyper-responsiveness [31]. Some of the common physiological responses to a stressor are summarized in Table 2 [32]. Elevated NE levels in tissue or plasma can directly affect cell types including

cancer cells and the following sections summarize some of the most important biological effects of catecholamines.

Table 2: Physiological response to stressor [32]

Oxygen and nutrients directed to the CNS and stressed body site(s)	Detoxification from toxic products
Altered cardiovascular tone, increased blood pressure and heart rate	Inhibition of growth and reproduction
Increased respiratory rate	Inhibition of digestion-stimulation of colonic motility
Increased gluconeogenesis and lipolysis	Containment of the inflammatory/immune response

1.3.1 Clinical Depression and CES-D score

Depression is one of clinical manifestations of chronic psychological stress. By definition, depression or major depressive disorder (MDD) refers to a mental condition characterized by lack of motivation and low mood that is persistent across all situations for at least 2 weeks [33]. Among all mental disorders, National Institute of Mental Health estimates MDD as the most common and estimate about 6.7% of adult population had at least one major depressive episode in 2015. National Comorbidity Survey was the first large scale epidemiological study that looked at prevalence of MDD in Americans. The study concluded that there was a 17% lifetime prevalence, and 6.7% of the population showed symptoms of MDD in the past 28 days [34]. The same study also

showed prevalence was at 21.3% in women and 12% in men. MDD also accounts for the highest amount of years lost due to disability (YLD) among mental disorders. YLDs are commonly used to define how ailments and diseases affect workforce and its impact on economy. It is calculated by multiplying the prevalence of a disorder (physical or mental) by the short- or long-term loss of health associated with that disability (the disability weight) (figure 7).

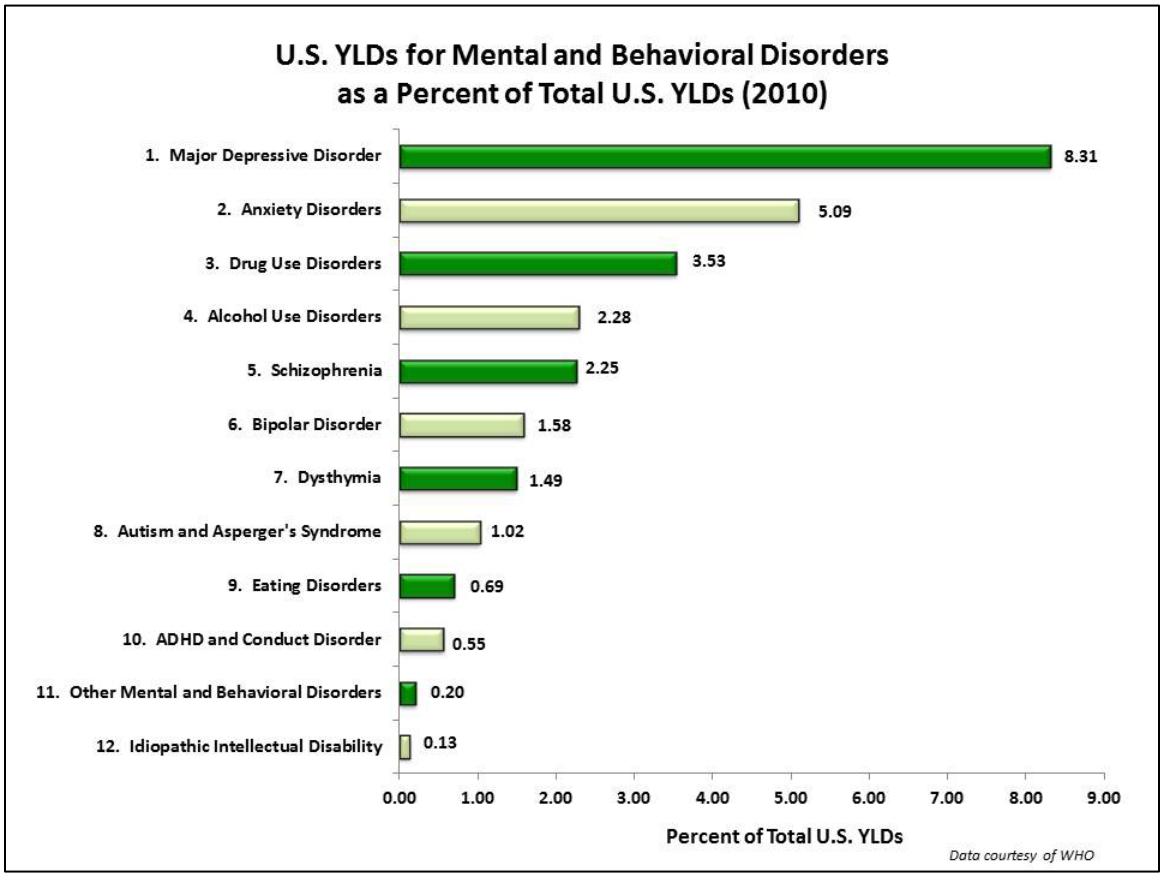

Figure 7: Effect of MDD on YTD. National Institute of Mental Health, Retrieved January 2017. https://www.nimh.nih.gov/health/statistics/disability/us-ylds-contributed-by-mental-and-behavioral-disorders.shtml

While certain genetic factors and biochemical factors might predispose a patient to MDD, psychological stressors are the major cause. In the clinic, MDD is

assessed by Center for Epidemiological Studies-Depression (CESD) screening diagnosis. It was first published by Radloff in 1977 as a 20-point questionnaire used to quantify depressive symptoms [35]. This is a self-report questionnaire that is used broadly in epidemiological studies to assess clinical depression it has shown good reproducibility and reliability across several populations [35]. This reliability has been attributed to the nature of the questionnaire that focuses primarily on psychological and cognitive effects of depression and not physical parameters such as pain, loss of appetite etc. [35, 36]. A cutoff of 16 is considered to a good indicator of risk of clinical depression with good specificity and sensitivity. A correlation between cancer and high CESD have been shown in other cancers such as ovarian, testicular, lung cancers [37-39].In a large scale study comparing psychological evaluations between healthy and breast cancer patients, there was a significant increase in CESD scores among cancer patients. Not surprisingly, there was an increase in CESD score among patients prior to initiating treatment and midway through their treatment (table 3) [40].

Mean CES-D scores of the patient group and healthy comparison group			
	Patient group	**Healthy comparison group**	**F**
Time 1	10.9 (sd = 8.9)	8.1 (sd = 7.0)	4.71
Time 2	12.8 (sd = 10.2)	7.8 (sd = 7.5)	11.72

Table 3: Differences in CESD Score among healthy and breast cancer patients [40] (D. Hann, K. Winter, P. Jacobsen, Measurement of depressive symptoms in cancer patients: Evaluation of the center for epidemiological studies depression scale (Ces-d), Journal of Psychosomatic Research, 46 (1999) 437-443.) Journal

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In ovarian cancer, Huang et al studied ADRB status and depression symptoms on survival [41]. For this study, the authors studied 237 patients from the Nurse Health study. The anxiety and depressive symptoms were assessed using Crown-Crisp Experiential Index (CCEI) and ADRB2 status assessed by immunohistochemistry. Tumors from 19% of patients stained positive for ADRB2 and these were also had significantly high levels of anxiety, depression symptoms compared with those patients bearing ADRB negative tumors. Dysregulation of the autonomic nervous system (ANS) is also associated with depressed patients with coronary heart disease.

1.4 Effects of Stress on Disease

Chronic stress has adverse effects on a number of diseases and this is summarized in the review below (figure 8). The following sections elaborate further some of the most important effects of adrenergic signaling and NE.

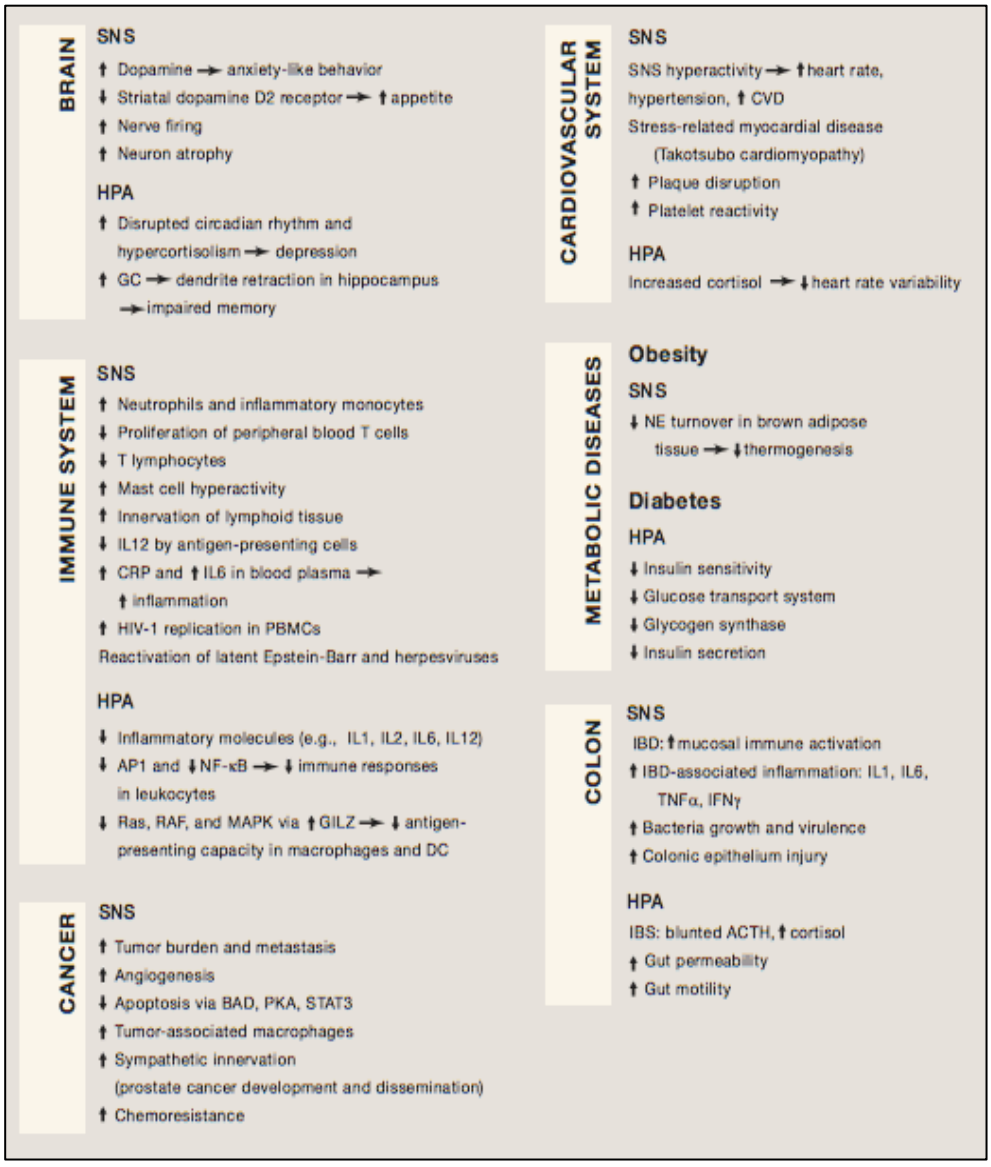

Figure 8: Effects of chronic stress on disease [42] (A.S. Nagaraja, N.C. Sadaoui, P.L. Dorniak, S.K. Lutgendorf, A.K. Sood, SnapShot: Stress and Disease, Cell Metab, 23 (2016) 388-388 e381.) Cell metabolism by ELSEVIER. Reproduced with permission of ELSEVIER in the format Thesis/Dissertation via Copyright Clearance Center.

Some of the earliest studies on effects of chronic stress were done to evaluate its adverse effects on cardiovascular disease. Chronic stress effects on

cardiovascular tissue can occur in different ways (figure 9). Plasma levels of NE (which is used as a biochemical indicator of adrenergic stimulation) is associated with mortality and cardiovascular outcomes [43]. High levels are associated with left ventricular dysfunction, high sympathetic activity in congestive heart failure [44-46]. Stress-induced endothelial dysfunction can be attributed to reduced vasodilation and accelerated atherosclerotic processes [47]. NE can also activate the renin-angiotensin-aldosterone system (RAAS) and this pathway is activated in patients with depressive symptoms [48-50]. Activation of aldosterone and mineralocorticoid receptors can result in increased pro-inflammatory cytokine production such as MCP1, MIF1a, and IL8 among others. NE and SNS activation can increase atherosclerotic plaques, several mechanisms such as increased cholesterol, increased macrophages, increased leukocytes, decreased vascular smooth muscle cells and increased likelihood of plaque rupture [51-53]. Cardiac tissue has an abundance of ADRB1 receptors, and resting heart rate is higher in depressed patients irrespective of their hypertensive status [54].

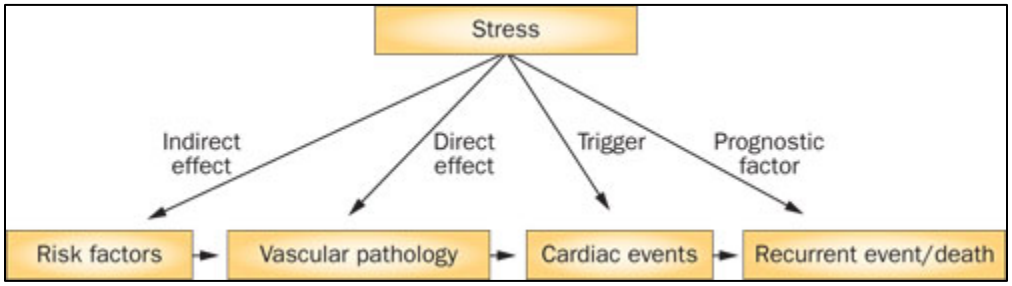

Figure 9: Chronic stress and cardiovascular disease [55] A. Steptoe, M. Kivimaki, Stress and cardiovascular disease, Nat Rev Cardiol, 9 (2012) 360-370.) (Nature Reviews Cardiology by World Heart Federation Reproduced with permission of

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Takatsubo cardiomyopathy commonly referred to as the broken-heart syndrome is a temporary and reversible heart condition triggered by sudden severe or emotional stress (such as death of a loved one, natural calamities, motor vehicle accident etc) [56, 57]. This is foun most commonly in postmenopausal women, and specific mechanisms are unknown. The sudden surge in catecholamine signaling triggers left ventricular dysfunction that can then no longer contract completely, resulting in the enlargement of the left ventricle to resemble a Japanese pot.

1.4.1 Effects of chronic stress on immune system

Chronic stress-induced immune dysregulation can have significant deleterious effects on health including blunted response to vaccination, slow and compromised wound healing, reactivation of latent viruses such as Herpes Simplex or Epstein–Barr virus (EBV), and increased risk other infectious diseases [58-60] (figure 10). Several human studies have shown that physiological stress impairs wound healing. Wound healing is a multiple step processes initiated by an inflammatory response, which is sequentially followed by proliferation (rebuilding tissue) and maturation (tissue remodeling phases). The first phase of wound healing requires an inflammatory response to recruit immune cells such as phagocytes and fibroblasts by producing chemokines and cytokines and this is important because when this stage is impaired, healing is disrupted [61, 62]. Proinflammatory cytokines such as IL6, TNF-a, IL1 are

decreased in the wound site during psychological stress and this can delay healing. Psychological stress, anxiety, depression have all been attributed to decreased adaptive cell immunity involving both B-cells and T-cells thereby increasing clinical risk of diseases [60, 63]. Psychological stress mediated reduced cellular immunity can lead to increased recurrence of viral infections including the herpes viruses, influenza, Hepatitis B and Epstein–Barr virus (EBV) by reactivating latent viruses and altering immune responses to the virus [64-67]. Mechanistically, this is due to the shift in the cytokine profile from a Th1 toward a Th2 profile that decreases cytokines important to mount a response against viruses [27]. Chronic stress is also associated with reduced NK cell activity in both human and animal models [68, 69].

Chronic psychological stress has been shown to be an aggravating factor in inflammatory bowel disease (IBD). Catecholamines can change gut permeability and bacteria adherence to mucosal surface [70]. Intestine is highly innervated with sympathetic neurons and increased NE activated mast cells in the gut mucosa driving inflammation and production of pro-inflammatory cytokines [71].

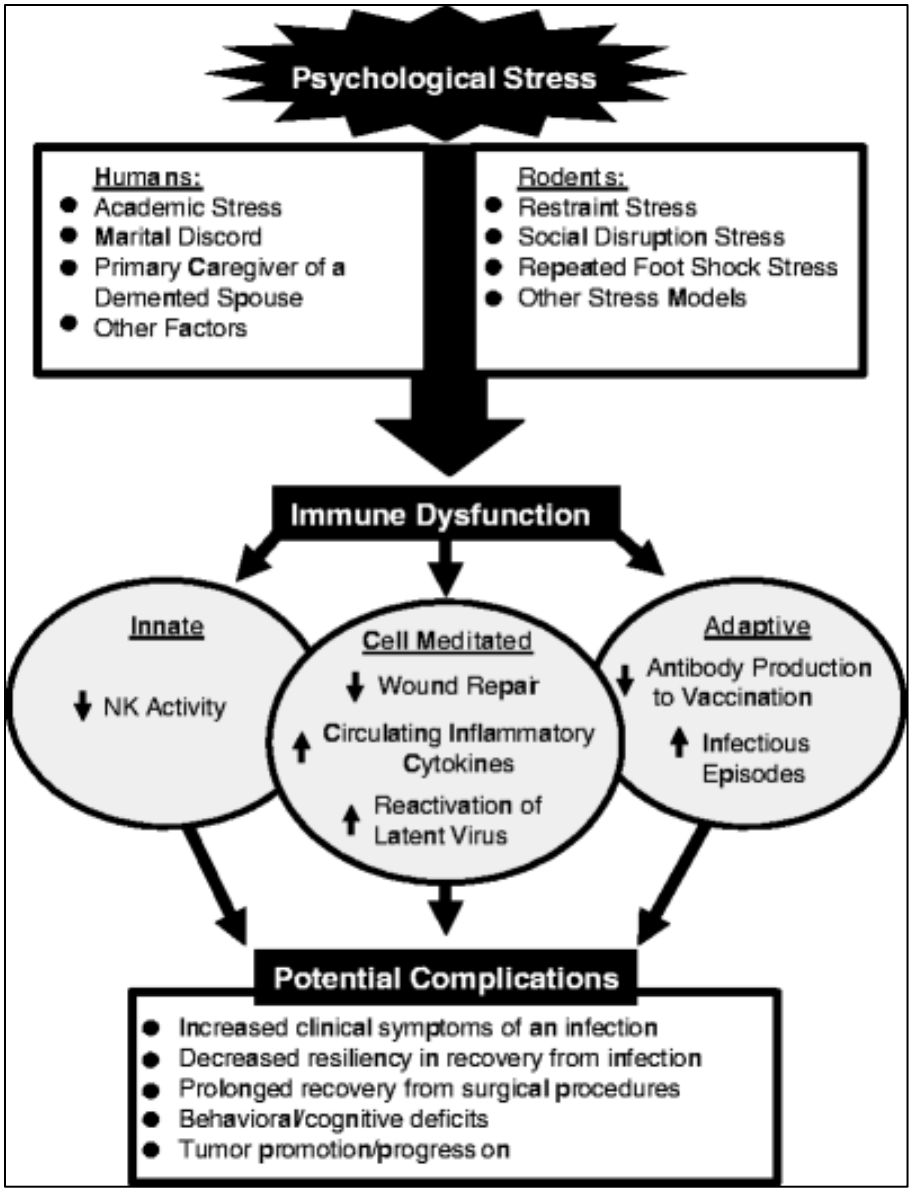

Figure 10: Stress effects on Immune function [58] (J.P. Godbout, R. Glaser, Stress-Induced Immune Dysregulation: Implications for Wound Healing, Infectious Disease and Cancer, J Neuroimmune Pharm, 1 (2006) 421-427.) Journal of neuroimmune pharmacology by Society on NeuroImmune Pharmacology Reproduced with permission of SPRINGER NEW YORK LLC in the format Thesis/Dissertation via Copyright Clearance Center.

1.4.2 Effects of chronic stress on viral biology

Norepinephrine and cortisol have several effects on viruses including accelerated replication, reactivation of latent viruses and reduced response to therapeutics. In a prospective study, a link was found between susceptibility to the common cold and levels of psychological stress [72]. Cortisol is associated with enhanced ability of HIV viruses to infect lymphocytes as well as immune suppression. Norepinephrine is shown to activate HIV replication in vitro and reduced response to antiviral therapy [73]. Increased HIV replication upon norepinephrine treatment was due to activation of protein kinase A (PKA) downstream of adrenergic receptors. This resulted in altered cytokine production by HIV-infected PBMC, particularly decreased production of IL10 and IFN-gamma [74]. Herpes simplex virus (HSV) can remain latent for many years in sensory and peripheral ganglia and stress hormones such as norepinephrine can decrease latency of disease. Mechanistically, this is because norepinephrine can decrease production of interferon-gamma which activate macrophages that fight HSV infection [75].

Unpublished data from our lab shows that NE can drive HPV-driven cervical cancers by increasing tumor growth and metastasis. Treating tumor-bearing animals with propranolol abrogated these effects.

1.4.3 Effects on nervous system

In patients with MDD studies looking at structural differences and functional MRI (fMRI) in the brain suggest several changes in the activity of specific areas in the brain. For example, there is hyperactivity in the ventromedial prefrontal cortex

and lateral prefrontal cortex that modulates pain/aggression and assesses risk respectively [76, 77] (figure 11). On the other hand, dorsolateral prefrontal cortex, that is required for sustained memory processed, show decreased activity [78]. The amygdala is located deep in the brain's medial temporal lobe and is the central component in the neural circuitry that processed emotions and pain. Amygdala is heavily innervated by adrenergic system and NE levels increase during stress [79]. Under emotional distress, amygdala is one of the main targets of NE during pain modulation [79]. This has been shown to predispose individuals to drug-seeking as well increased likelihood of drug relapse [80]. The hippocampus is involved with memory and learning, and chronic stress and stress hormones have been shown to impair both [81]. Clinical data shows that patients exposed to severe stressful events (such as those diagnosed with PTSD) show to a smaller hippocampal volume that can correlate to impaired memory [82]. Long term potentiation refers to a neuronal process caused by repeated and frequent activation of afferent fibers synapses that can strengthen memory formation. Preclinical studies using rats have shown that chronic stress can alter LTP in 2 regions of the hippocampus, CA3 and dentate gyrus [83]. In preclinical models, stress impairs memory by altering ensuing synaptic plasticity

and firing properties of hippocampal neurons [81]. In preclinical models, chronic stress leads to depression-like behavior, including heightened fear learning in presence of prey-scent, increased helplessness, fatigue and decreased motivation for food and other rewards [84].

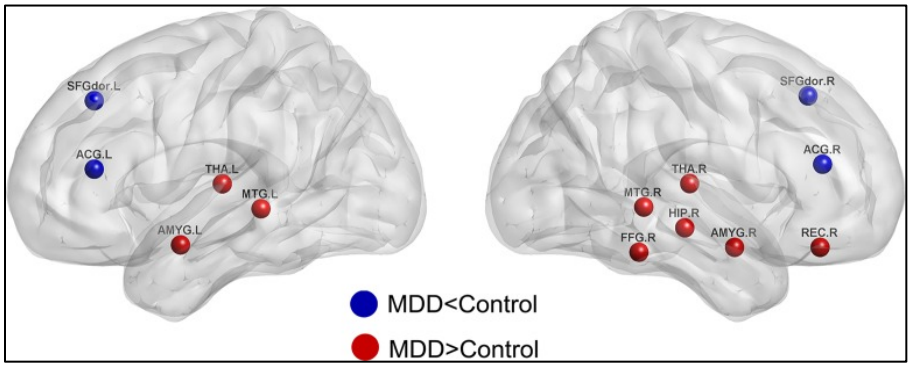

Figure 11: Regions exhibited significant between-group differences in regional nodal parameters between control and patients with MDD [77] Figure as originally published M. Ye, T.L. Yang, P. Qing, X. Lei, J. Qiu, G.Y. Liu, Changes of Functional Brain Networks in Major Depressive Disorder: A Graph Theoretical Analysis of Resting-State fMRI, PloS one, 10 (2015).

1.5 Chronic stress and cancer

1.5.1 Clinical studies

Psychological stress and clinical depression can affect outcomes such as survival and relapse in several cancers such as lung, ovarian, breast, colon and renal carcinomas [85-88]. Studies have also shown that clinical depression is common among patients with melanoma, pancreatic, prostate, ovarian, breast, or lung cancers [89-93]. Ovarian and breast cancers are the most widely studied cancer for effects of depression, social support or other biobehavioral factors. In

a study by Lutgendorf et al, of 68 ovarian cancer patients, low social support was associated with elevated intratumoural NE level whereas there were no differences in plasma levels [85, 86]. In another epidemiological study, Costanzo et al. showed that in a cohort of 61 ovarian cancer patients, patients with low social support also had higher levels of interleukin 6 (IL-6) in blood and ascites [94]. A history of major depression is also associated with elevated plasma IL6 levels in breast cancer. Renal cell carcinoma patients with high CESD score show a prominent pro-inflammatory signature in PBMC including high expression of Cox2, IL6 and other markers [86].

Conversely, incidental usage of a beta-blocker is shown to have survival benefits in several studies [95, 96] (figure 13). In one of the most comprehensive studies, 3561 patients with high-risk prostate cancer, of whom 1115 patients had used a beta-blocker before and after diagnosis, use of a beta-blocker affected several aspects of disease. It was significantly associated with a delayed onset of disease, reduced risk of mortality from prostate cancer, a lower Gleason score and a lower rate of distant metastasis [97]. Aspirin is a widely used NSAID that was shown in this study to have an additive survival benefit when combined with a beta-blocker. On a molecular levels, expression of ADRB2 in oral squamous carcinoma cells was associated with greater tumor size, higher rate of lymph node metastasis and advanced clinical stage in patients [96]. A retrospective study done on patients with epithelial ovarian cancer showed that use of a beta-blocker increased both progression-free and overall survival when compared to patients who had never used a beta-blocker. Several clinical studies support the

beneficial role of beta-blockers in breast cancer as well. In a cohort of 466 breast cancer patients, 43 patients who were on a non-specific or ADRB1-targeted beta-blocker (atenolol) for hypertension had significantly prolonged survival and lower rates of metastasis as well as recurrence [98, 99]. In a contradictory study, Barron et al. grouped breast cancer patients into those not on a beta-blocker, those on atenolol (beta-1 selective) and those on propranolol (non-selective beta blocker) [98]. Significant survival benefit was seen only among patients who were on propranolol, and not on those on atenolol. Past therapy with propranolol was also associated with lower tumor grade and lower incidence of metastasis than in patients not on a beta-blocker. Use of a beta-blocker was associated with reduced risk of death in patients with malignant melanoma. In a recent retrospective study in non–small cell lung cancer, use of a beta-blocker (ADRB1 specific or non-selective) during radiation therapy was associated with significantly better rates of overall survival, distant metastasis-free survival and progression-free survival. In infantile haemangioma, propranolol is one of the most promising options for therapy, as it decreases expression of VEGF, which is vital in progression of this disease [100]. Propranolol is not approved by FDA for pediatric cancers, but it has been used to treat cardiac ailments in children and is documented to be well tolerated, with side effects that are seldom life threatening. There are currently clinical trials underway to assess propranolol (NCT01908972, NCT01211080), nadolol (NCT01010308), timolol (NCT02731287) and other beta-blockers for treatment of hemangioma. Beta-

blocker usage is associated with decreased cancer-related mortality, using data from the FDA Adverse Event Reporting System (figure 12) [101].

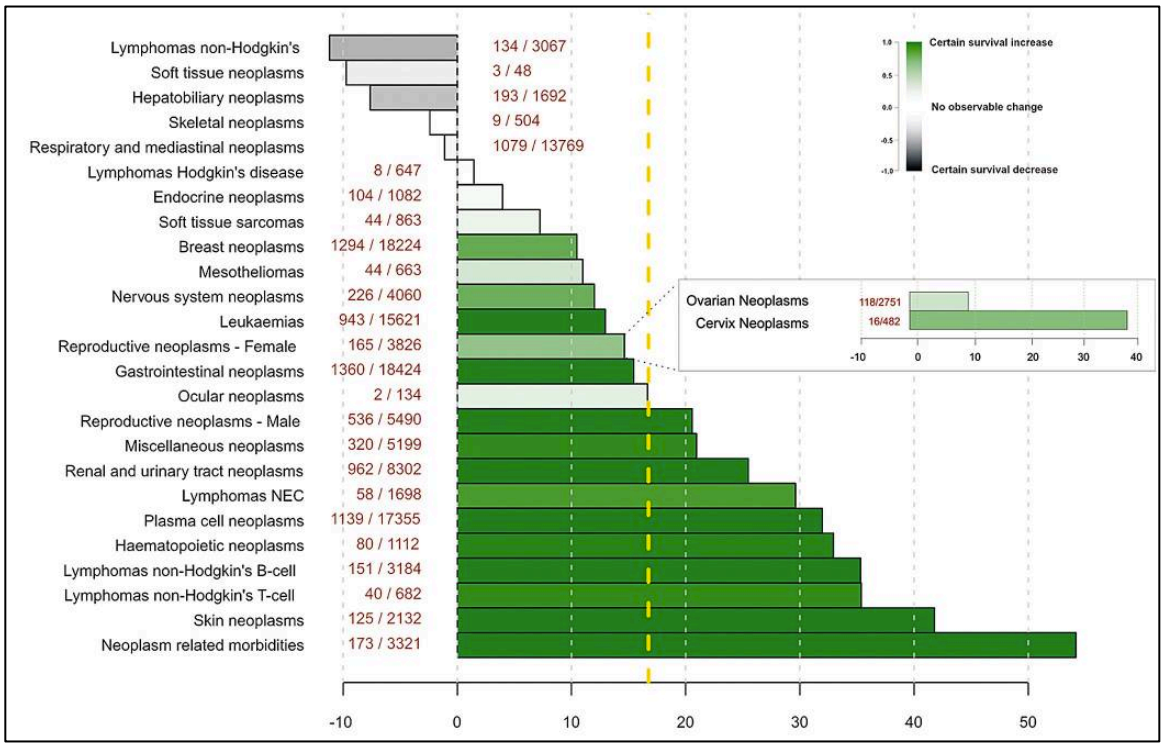

Figure 12: Effect of beta-blocker on cancer-related mortality [101]. (G.N. Armaiz-Pena, J.K. Allen, A. Cruz, R.L. Stone, A.M. Nick, Y.G. Lin, L.Y. Han, L.S. Mangala, G.J. Villares, P. Vivas-Mejia, C. Rodriguez-Aguayo, A.S. Nagaraja, K.M. Gharpure, Z. Wu, R.D. English, K.V. Soman, M.M. Shahzad, M. Zigler, M.T. Deavers, A. Zien, T.G. Soldatos, D.B. Jackson, J.E. Wiktorowicz, M. Torres-Lugo, T. Young, K. De Geest, G.E. Gallick, M. Bar-Eli, G. Lopez-Berestein, S.W. Cole, G.E. Lopez, S.K. Lutgendorf, A.K. Sood, Src activation by beta-adrenoreceptors is a key switch for tumour metastasis, Nature communications, 4 (2013) 1403.) Nature communications by Nature Publishing Group. Reproduced with permission of Nature Publishing Group in the format Republish in a thesis/dissertation via Copyright Clearance Center.

1.5.2 Preclinical models for chronic stress

Preclinical models of chronic stress are difficult to recapitulate when we try to mimic all the psychological workings of chronic stress in humans. Biobehavioral symptoms and signs of clinical depression such as suicidal thoughts, low self-esteem and mood swings are impossible to obtain in preclinical models. Hence, scientists use predicted molecular and physiological responses to depression to evaluate animal models (table 4) [102]. Restraint stress is one of the most widely used models to mimic clinical depression for several reasons. This model enables reliable assessment of stress-related biobehavioral and biochemical changes in laboratory animals. The test is also easy to perform and has high reproducibility. Restraint stress enables us to study both acute and chronic stress, and produce a mental stress to animals that have low adaptability. In addition, this test is also painless for the animals, and would not trigger other pathways (such as hypothermia, pain) that might contribute to tumor growth. Some of the other widely used preclinical models are summarized in the table below.

Table 4: Animal models of chronic stress

Model		**Advantages**
Tail Suspension	Mice suspended from tail, time to reach up measured	Easy to perform
Wet bedding	Bedding of cage kept damp	Easy to perform
Learned helplessness	Exposure to uncontrollable and unpredictable stressors Foot shock	Easy to perform

Maternal deprivation	Early stage stress model	Induces anxiety symptoms
Forced swim test	Model for behavioral despair where time to escape is measured	Easy to perform

1.5.3 Effects on tumor initiation

Very little preclinical evidence shows a role for stress in driving tumor initiation. Saul et al, used SKH1 which is a melanoma model susceptible to UV-induced tumors to study effects of chronic stress on tumor initiation. Restraint stressed animals had a shorter time to occurrence of the first tumor, which can be interpreted as shortened latency to disease as true initiation. The stressed animals also had high levels of proinflammatory cytokines and reduced T-helper cells compared to controls [103]. In another model of melanoma, chemically induced tumors using 7,12-dimethylbenz(a)anthracene (DMBA) alone (topical), and DMBA-12-O-tetradecanoylphorbol-13-acetate (TPA) after chronic unpredictable stress increased tumor incidence and tumor burden [104]. Innervation of peritumoral or intratumoral regions can produce catecholamines and is increasingly shown to play roles in tumor progression in prostate, pancreatic and ovarian cancers [105-108]. However, definite causation of tumors owing to increased adrenergic stimulation is not well studied.

1.5.4 Direct effects on cancer cells

Some of the first studies looking at the molecular effects of norepinephrine on cancer cells were done in ovarian cancer. Using restraint stress, Thaker showed

that chronic restraint stress can lead to increased tumor growth and metastasis [109]. Several cancer cells are positive for ADRB receptors. Activation of these receptors activates transcription factors such as CREB, PKA and Nf-kB. Thaker and Shahzad showed in independent studies that chronic stress increases levels of VEGF and IL8 in an ADRB2 dependent manner thereby promoting angiogenesis [109]. Sood et al also showed that NE and ADRB2 mediated signaling can promote cell survival during anoikis that can further help disseminated cells to metastasize effectively to distant organs [110]. Armaiz-Pena et al, showed that NE can potently induce metalloproteinases (such as MMP2 and MMP9), Src, and FAK activation in cancer cells and contribute to metastasis [101]. Hassan et al showed that NE could activate anti-apoptotic signaling in a PKA-dependent manner and promote cell survival in a model of prostate cancer [106]. In vitro studies in ovarian, colon, melanoma and pancreatic cancer cell lines have shown NE can potently increase invasion and migration of cells [111-114]. NE can also promote inflammation by increasing PGE2 levels in an ADRB2-Nf-kB-PTGS2 axis in ovarian cancer [115]. NE can also blunt effects of chemotherapy and anti-angiogenic therapy. Kang et al, showed that NE can reduce effects of platinum and paclitaxel therapy in ovarian cancer via NE-ADRB2-Jnk-DUSP1 pathway [116]. Preclinical models of lung and colorectal cancers show that chronic stress can decrease the therapeutic benefit of sunitinib by increasing VEGF, IL8 and these effects can be blocked by propranolol. [117, 118]. In preclinical in vivo and in vitro studies, blocking NE

using propranolol or specific beta-blockers can abrogate tumor growth, metastasis or angiogenesis [107, 108].

1.5.5 Effects on stromal cells

There are an increasing number of studies that have shown that stromal factors contribute significantly to all aspects of tumor progression including growth, metastasis, immune suppression, angiogenesis, resisting cell death [119]. There are several infiltrating immune cells such natural killer cells, tumor associated macrophages, and T and B-lymphocytes. Tumor associated macrophages produce VEGFA, an important factor in promoting angiogenesis, and can also produce several proinflammatory cytokines. NK cells are cytotoxic lymphocytes of the innate immune system that produce cytotoxic granules containing perforin and various granzymes upon activation and cause perforations and apoptotic death of tumor cells. Impaired NK cells or NK cell deficiency is associated with increased incidence of cancer in preclinical models and human studies [120]. T cells in tumors have been studied intensively over the past years and with immunotherapy, is emerging as one of the main targets to treat various cancers. Antibodies against checkpoint inhibitors such as CTLA4 or PD1 have shown complete or partial response in renal cell carcinoma, head and neck cancer, melanoma, lung cancer among others [121]. In several cancers, low cytotoxic T-cell infiltration (CTL) within tumors is associated with low survival. The proinflammatory microenvironment is considered to one of the most important impediments to T-cell infiltration [121]. Tumor associated endothelial cells have weak intercellular connections resulting in a leaky vasculature that can contribute

to metastasis. In addition they can a) produce several growth factors, b) engage leukocytes and platelets and play a role in trafficking of immune cells and c) produce factors such as PGE2, VEGF that impede T-cell infiltration into tumors [122]. Some of the other cell types and their most important roles are summarized in figure 13.

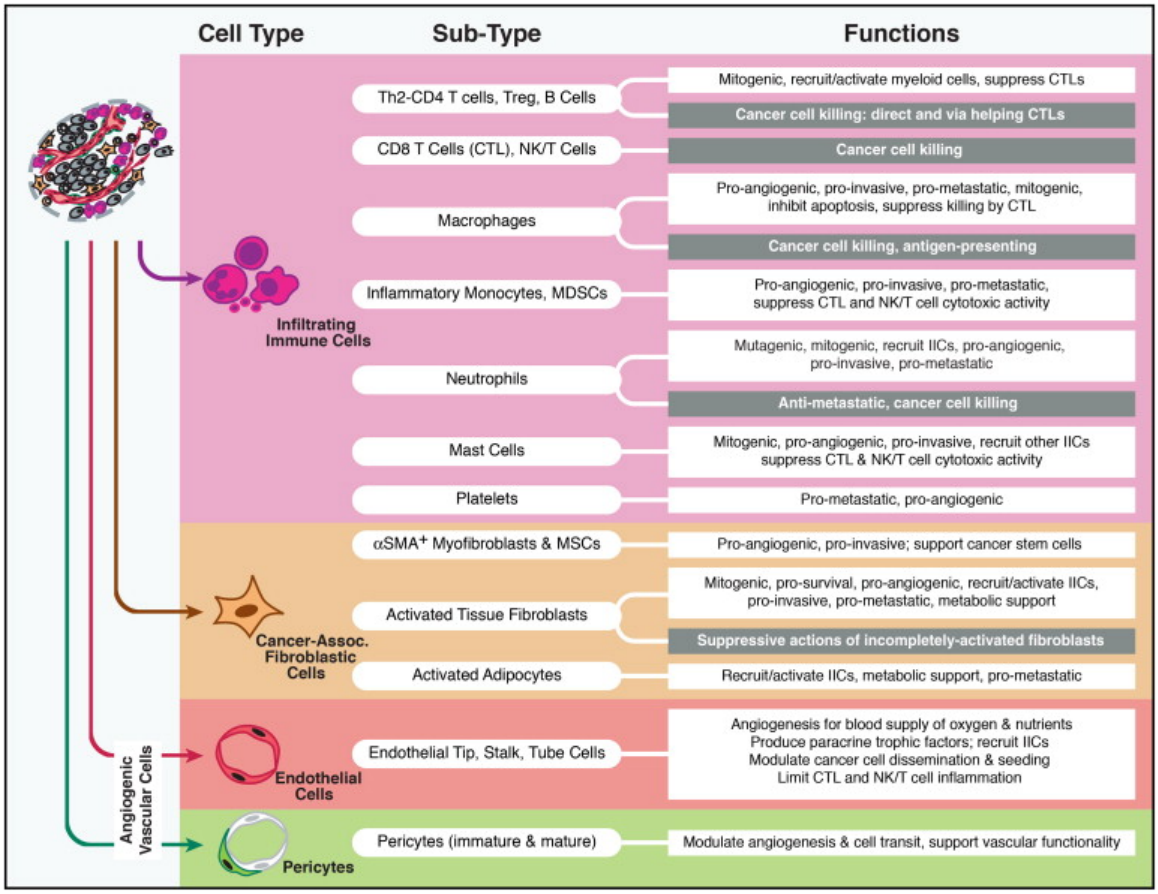

Figure 13: Stromal contributions to tumor progression.[119] (D. Hanahan, L.M. Coussens, Accessories to the crime: functions of cells recruited to the tumor microenvironment, Cancer Cell, 21 (2012) 309-322.) Cancer cell by CELL PRESS. Reproduced with permission of CELL PRESS in the format Thesis/Dissertation via Copyright Clearance Center.

Effects of NE on stromal cells have not been as well studied as tumor cells (figure 14). Armaiz-Pena et al showed that NE induced MCP1 production by cancer cells can promote tumor-associated macrophages in ovarian cancer [123]. Sloan et al, showed the NE-induced elevations in tumor-associated macrophages increased breast cancer metastasis [124]. Ben-Eliyahu et al, showed that in models of breast cancer and leukemia, NK cell activity was suppressed during chronic stress and could attribute to increased tumor growth [125, 126]. A study in brain cancer shows tumor endothelial cells are positive for adrenergic receptors and can promote tubulogenesis and MMP9 secretion and propranolol can block this effect [127, 128]. Infantile hemangioma is an endothelial-derived tumor in which the first line of therapy is propranolol [129].

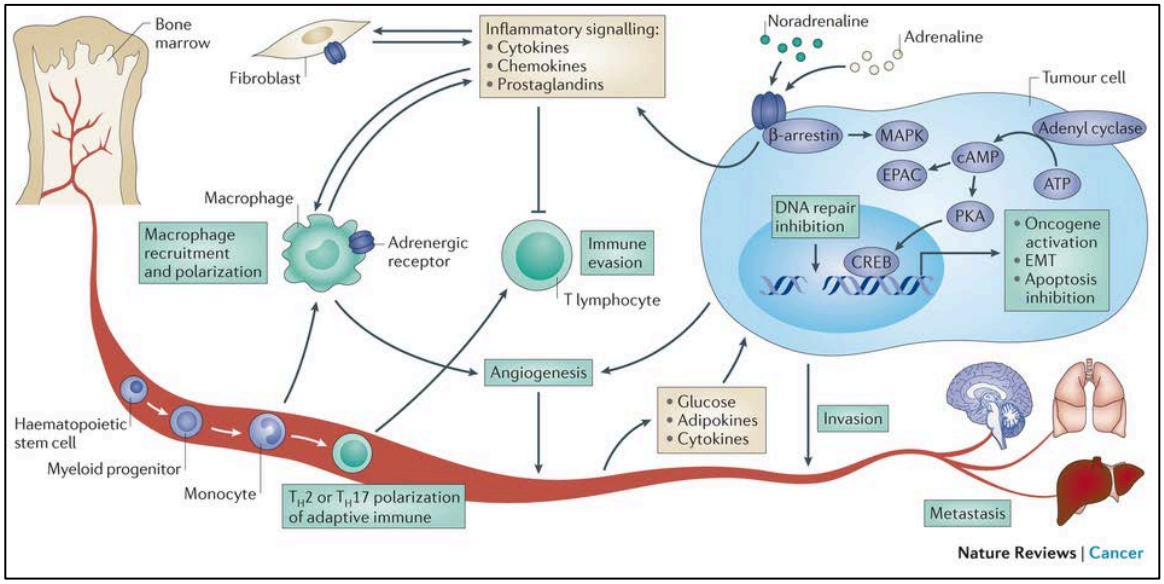

Figure 14: Effects of catecholamines on cancer and stromal components [88] (S.W. Cole, A.S. Nagaraja, S.K. Lutgendorf, P.A. Green, A.K. Sood, Sympathetic nervous system regulation of the tumour microenvironment, Nature reviews. Cancer, 15 (2015) 563-572.) Nature Reviews Cancer by NATURE PUBLISHING

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1.6 Cancer associated fibroblasts

A typical tumor contains endothelial cells, pericytes, immune cells such as macrophages, T- and B-cells, cancer-associated fibroblasts, adipocytes and other non-cellular components such as extracellular matrix and basement membrane. Among them CAFs are usually the most abundant stromal cell in tumors and play important roles in tumor progression. Once thought to be mere bystanders, CAFs are currently studied extensively due to emerging studies that show roles in chemoresistance, resistance to immunotherapy, metastasis and invasion [130-132].

1.6.1 Definitions

CAFs can also be referred to as tumor-associated fibroblasts, activated myofibroblasts or simply activated fibroblasts (figure 15) [133, 134]. Resident fibroblasts in most normal tissue are resting or quiescent, however they become ‘activated’ during tissue injury and during presence of tumor cells. The cues that trigger activation during wound healing is known but cues during tumor growth are not studied well. Activated fibroblasts in both cases however show similar characteristics in that they produce a number of cytokines, increase collagen production, and show presence of certain markers of activated CAFs [134]. Functionally, these can have several growth-promoting effects on a tumor.

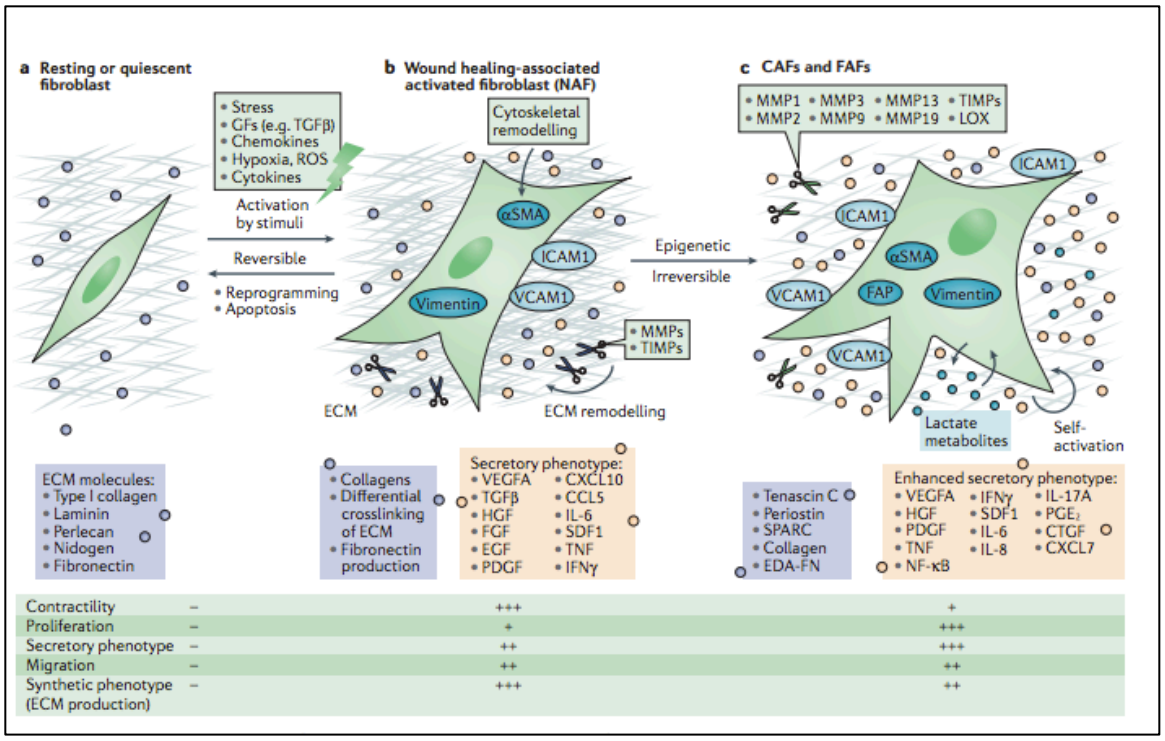

Figure 15: Activation of fibroblasts [133] (R. Kalluri, The biology and function of fibroblasts in cancer, Nat Rev Cancer, 16 (2016) 582-598.) Nature Reviews Cancer by NATURE PUBLISHING GROUP. Reproduced with permission of NATURE PUBLISHING GROUP in the format Republish in a thesis/dissertation via Copyright Clearance Center.

1.6.2 Origin and markers

There is wide heterogeneity in CAFs and hence, there are several markers that are taken together to identify them. Alpha-Smooth muscle actin (a-SMA) is the most widely used CAF marker to identify tumoral fibroblasts and other markers include fibroblast-activated protein (FAP), fibroblast-specific protein-1 (FSP1/S100A4), neuron-glial antigen-2 (NG2) and PDGF β-receptor. Several of these markers are common with other cells within tumors such as smooth muscles (a-SMA), pericytes (NG2) and tumor cells (PDGFR-b). Morphologically

similar CAFs may not stain positive for the same markers, and hence scientists believe that there are a subset of CAFs in tumors [131, 133].

CAFs can also be derived from several sources, but primarily from 2 sources: residual fibroblasts or recruitment of mesenchymal stem cells into tumors (figure 16). Several studies of colon, breast and lung cancers suggest that local activation of fibroblasts contributes to CAFs in tumors [135]. Marini et al, used a lethally irradiated RFP+ mice which were reconstituted with GFP+ bone marrow to study local and bone-marrow derived stromal components in ovarian cancer [136]. Using several markers for CAFs such as a-SMA, NG2, FSP and FAP, they showed that a-SMA+ and NG2+ CAFs were derived from local tissue. FSP+ and FAP+ cells co-stained with GFP indicating mesenchymal stem cell origin [136]. There is some evidence to show that epithelial cells exposed to reactive oxygen species or MMPs during fibrosis can transdifferentiate into a CAF-like cell, which can deposit ECM proteins and stains positive for a-SMA [137]. Preclinical studies using GEM models with tagged lineages show that endothelial cells can also show CAF-like phenotype in a process called EndMT (endothelial to mesenchymal transformation) in melanoma [138].

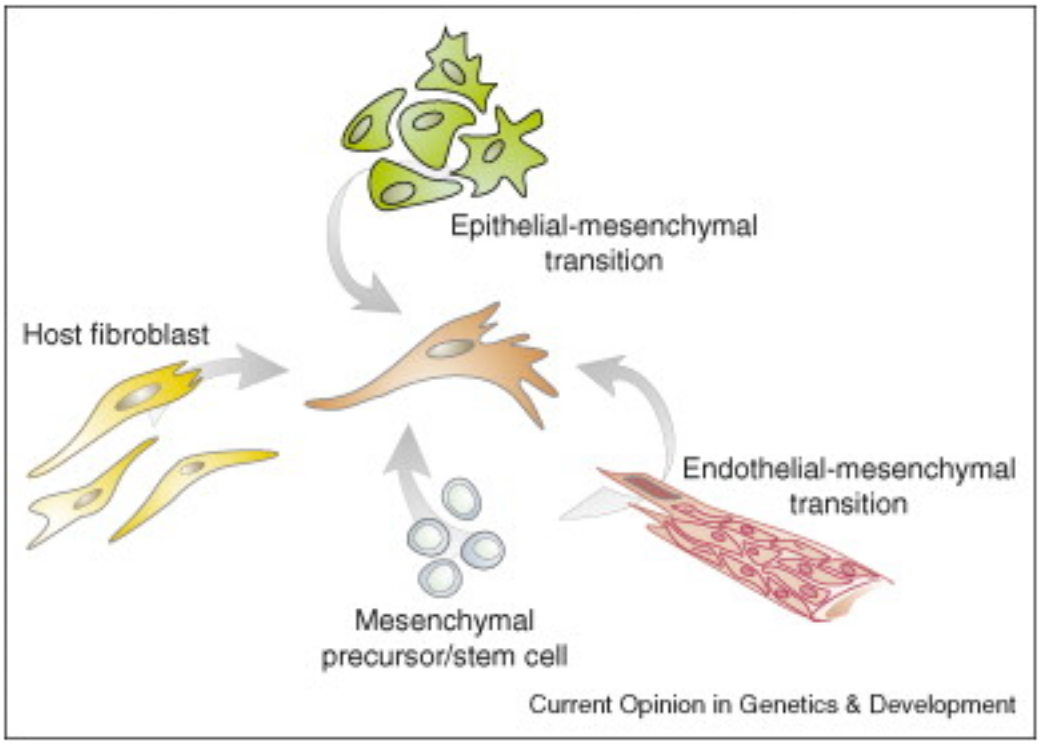

Figure 16: Origin of fibroblasts [132] (A. Ostman, M. Augsten, Cancer-associated fibroblasts and tumor growth - bystanders turning into key players, Curr Opin Genet Dev, 19 (2009) 67-73.) Current opinion in genetics & development by ELSEVIER LTD.. Reproduced with permission of ELSEVIER LTD. in the format Thesis/Dissertation via Copyright Clearance Center.

1.6.3 Functional roles in tumor microenvironment

Clinical studies have shown that higher levels of CAFs in colon, ovarian, lung, and breast cancers are all associated with decreased survival [139, 140]. The role of CAFs in driving tumor initiation and progression is multi-faceted (figure 17). When CAFs are co-incubated with premalignant cells, they can induce tumors and promote malignancy [141]. CAFs can directly affect and increase tumor growth. Activated fibroblasts produce a number of potent growth factor

such as HGF, IL6, EGF and CTGF [130, 131, 139, 140, 142, 143]. These can activate downstream signaling in tumor cells that promote both growth and metastasis. CAF-derived prostaglandin E2 can also reduce NK cell activity in tumors [144]. VEGF, TGFb, PDGFa derived from CAFs can stimulate endothelial cell growth promoting angiogenesis [145-147]. CAFs also produce several ECM proteins such as collagens that provide both signaling and scaffolding for tumor metastasis. MMPs and TIMPs help breakdown ECM proteins as needed for tumor growth. One of the biggest functions of CAFs is decreasing cell-mediated immunity. CAFs produce several extracellular factors such as GM-CSF, IL6, TGFb, CCL2, CCL5 that impede immune cell recruitment particularly T-cells.

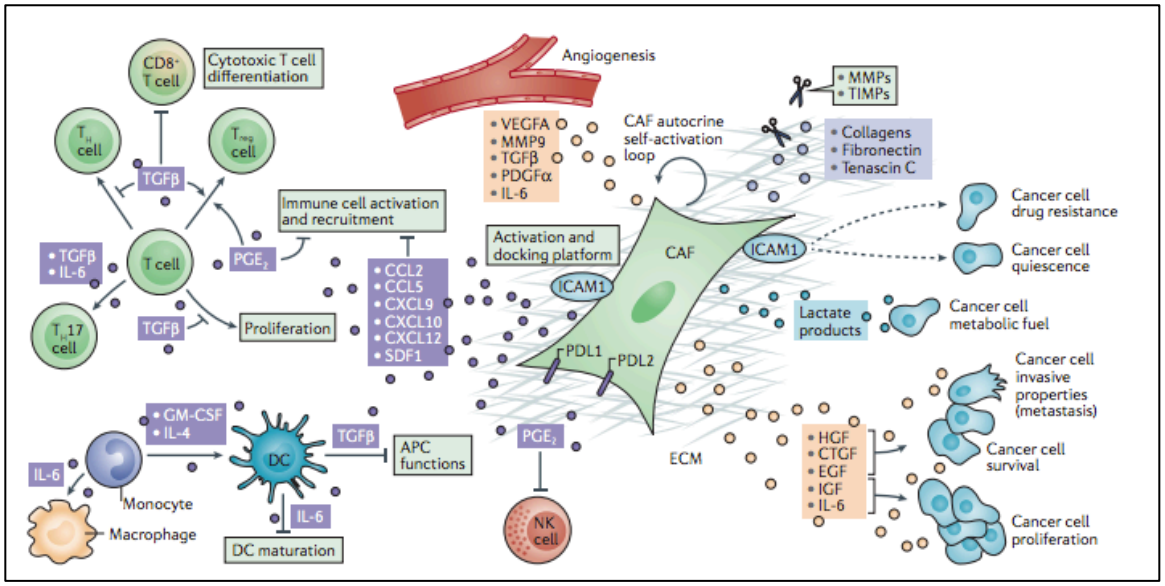

Figure 17: Functions of CAFs [148] (R. Kalluri, The biology and function of exosomes in cancer, J Clin Invest, 126 (2016) 1208-1215.) Nature Reviews Cancer by NATURE PUBLISHING GROUP. Reproduced with permission of NATURE PUBLISHING GROUP in the format Republish in a thesis/dissertation via Copyright Clearance Center.

1.7 Activin Signaling

Activins and inhibins are members of the TGF-b family of proteins. In normal tissue, it is primarily expressed reproductive tissue such as gonads, uterus and pituitary owing to its role in development of ovary, testis and follicle [149]. Inhibin, beta A is also shown to play a role in driving glucose stimulated insulin secretion in pancreatic beta-cells (figure 18) [149]. Inhibin, Beta A can drive SMAD signaling via heteromeric complexes and activation of AVR2a and ACVR2b receptors. Phosphorylation of SMADs 2/3 and SMAD4 complex leads to their translocation to the nucleus. Here the proteins can bind to specific sequences in the genome called SMAD binding element (SBE) and can drive transcription of several genes [150].

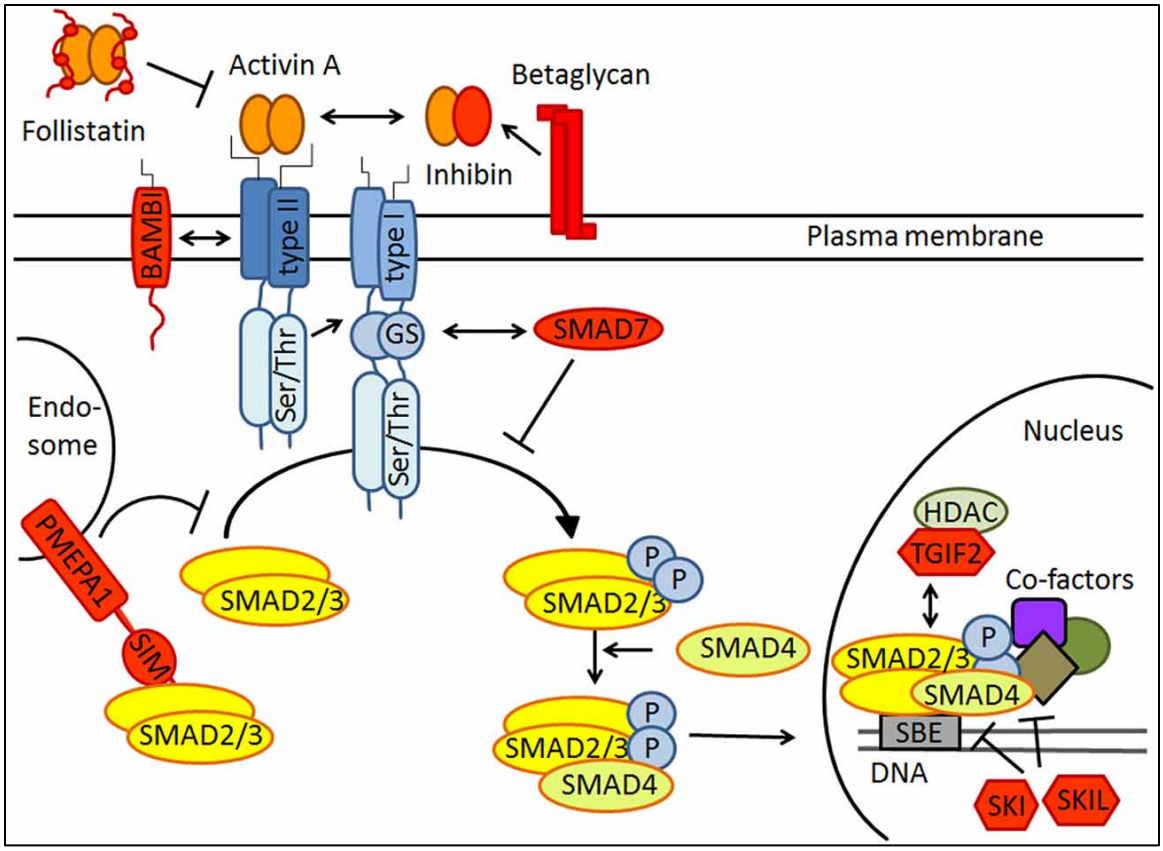

Figure 18: Inhibin, Beta A Signaling [150]. Figure as originally published in Metpally RPR, Nasser S, Malenica I, Courtright A, Carlson E, Ghaffari L, Villa S, Tembe W and Van Keuren-Jensen K (2013). Front. Genet. 4:20.

1.7.1 Inhibin, Beta A in cancer

Inhibin, Beta A (*INHBA)* is overexpressed in several cancers including prostate, ovarian, pancreatic, breast, colon and other cancers. Inhibin, Beta A is overexpressed in late-stage colon cancer patients compared to normal and early stage disease [151]. In breast cancer patients, Inhibin, Beta A was overexpressed invasive ductal carcinoma compared to normal breast tissue [152]. Immunohistochemical staining also showed elevated p-SMAD2 and p-SMAD3 in tumor samples compared to normal tissue. Recombinant Inhibin, Beta

A increased anchorage-independent growth in breast cancer cell lines MCF7 and MDA-MB-231 were used to study biological effects of Inhibin, Beta A [152]. *In vivo* studies using Inhibin, Beta A-overexpressing cell line showed increased tumor growth, metastasis and EMT. In prostate cancer, Inhibin, Beta A up-regulates expression of AKR1C3 (Aldo-Keto Reductase Family 1 Member C3) which is important for driving intratumoral levels of androgen. High levels of Activin was also associated with worse survival in prostate cancer [153]. In ovarian cancer, Inhibin, Beta A levels are higher in cancer compared to normal and plays a role in promoting invasion and migration of cancer cells [154].

2 Rationale and Significance

Several epideminiological and preclinical studies have shown that psychological factors can have adverse effects on human health. These include increased incidence of cardiovascular disease, inflammatory bowel disease, type 2 diabetes. Altered mental states such as depression or chronic stress, potently activates the sympathetic nervous system to produce catecholamines either by adrenal glands or local production by peripheral nerves. Recent evidence has shown that cancer cells have receptors for catecholamines, and can promote tumor growth, angiogenesis, inflammation, and macrophage infiltration. For this project, we sought to find a link between high levels of tumoral catecholamines and stromal components within ovarian tumors. I hypothesized that sustained adrenergic signaling during chronic stress can accelerates induction of a CAF-phenotype in ovarian tumors. My work demonstrates a) there is a prominent stromal signature in patients with high depressive symptoms compared to those with low depressive symptoms b) this is due to increased production of *INHBA (*Inhibin, beta A) by cancer cells after NE stimulation in an ADRB2-CREB axis and c) this results in activation of fibroblasts and these can increase collagen levels in the tumor stroma. Together these data represent the first demonstration that adrenergic signaling mediated Inhibin, beta A can drive CAF phenotype in ovarian tumors. This work provides evidence that blocking Inhibin, beta A in combination with beta-blockers may be a viable therapeutic target in chronically stressed patients in order to block the deleterious effects of chronic stress on patient outcome.

3 Methods

3.1 Cell lines

Skov3-ip1, A2780-ip2and HeyA8 ovarian cancer cell lines have been described previously [101]. They were maintained in RPMI-1640 medium supplemented with 15% fetal bovine serum and 0.1% gentamycin sulfate at 37°C. The ID8-ip1 murine ovarian cancer cell line was maintained in DMEM-high glucose medium supplemented with 10% fetal bovine serum, 0.1% gentamycin, and 0.1% insulin-transferrin sulfate [155]. NOF151 normal ovarian fibroblasts, whose derivation has been described before [139, 156], were a kind gift from Dr. Jinsong Liu. These cells were maintained in a 1:1 mixture of Medium 199 and MCDB 105 medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 5 µg epidermal growth factor. All cells were grown at 37°C in a humidified chamber with 5% CO_2.

For all *in vitro* experiments, cancer cells at 70% confluence were serum-starved overnight before treatment. NE (10 µM; Sigma-Aldrich, St Louis, MO, USA) was dissolved in sterile water just before it was added to the cells. Isoproterenol and terbutaline (Sigma-Aldrich) were used at published concentrations [115, 116]. Propranolol and butoxamine were purchased from Tocris (Bristol, UK) and used at published concentrations [115, 116]. Treatment with an antagonist, if pertinent, began 1 h before NE was added to the cell culture medium. Treatment with agonists was started at the same time as NE treatment. Genes were silenced by using a reverse-transfection protocol with RNAimax and control siRNA (sequence: UUAUGCCGAUCGCGUCACA Sigma-Aldrich), specific human INHBA siRNA (sequence: CCAACAGGACCAGGACCAA Sigma-Aldrich), human

ADRB2 siRNA (sequence: GCCATTACTTCACCTTTCA Sigma-Aldrich), human ACVR2a siRNA (sequence: GCUCCAACCUCGAAGUAGA Sigma-Aldrich) and human ACVR2b (CUCGACUUUGGGUUGGCCUU Sigma-Aldrich) according to the manufacturer's recommendations. Briefly, 40 nm siRNA was mixed with RNAimax transfecting agent and added to culture medium for 4 h. Fresh complete medium was added and cells were allowed to grow. Silencing was assessed at 48 h using quantitative reverse-transcriptase polymerase chain reaction (qRT–PCR) analysis.

3.2 Chronic stress model

All animal experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center, where the experiments were carried out. Experiments involving human cancer cell– and murine cancer cell–derived mouse tumor models were performed in 8- to 12-week-old female athymic nude and C57/B6 mice, respectively, obtained from Taconic Farms (Hudson, NY, USA*).* Adrenalectomized mice were received from Taconic Farms 3 days after surgery and were given an additional 10 days to recover before restraint stress started. Chronic stress was induced experimentally by using a restraint-stress procedure that has been previously described [109]. In brief, mice were enclosed in a movement-restricted space for 2 h daily for the duration of the experiment (figure 19).

Figure 19: Restraint system set up [109]. (P.H. Thaker, L.Y. Han, A.A. Kamat, J.M. Arevalo, R. Takahashi, C. Lu, N.B. Jennings, G. Armaiz-Pena, J.A. Bankson, M. Ravoori, W.M. Merritt, Y.G. Lin, L.S. Mangala, T.J. Kim, R.L. Coleman, C.N. Landen, Y. Li, E. Felix, A.M. Sanguino, R.A. Newman, M. Lloyd, D.M. Gershenson, V. Kundra, G. Lopez-Berestein, S.K. Lutgendorf, S.W. Cole, A.K. Sood, Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma, Nature medicine, 12 (2006) 939-944.) Nature medicine by NATURE PUBLISHING GROUP. Reproduced with permission of NATURE PUBLISHING GROUP in the format Republish in a thesis/dissertation via Copyright Clearance Center.

Tumor cells were injected intraperitoneally into mice in all groups a week after the stress procedure began (Skov3-ip1: 1 million cells/animal, HeyA8: 250,000 cells/animal, ID8-ip1: 2 million cells/animal). Mice were randomly assigned to groups (n=10/group), and siRNA (3.5 µg in DOPC) treatment was started 5 days after tumor cell injection and continued twice weekly for the duration of the experiment. Mice were treated by intraperitoneal injection with propranolol or a specific beta-blocker or adrenergic agonist daily for the duration of the experiment. The animals were sacrificed by cervical dislocation when they became moribund; the cadavers were examined for visible disease, and mouse

weight, tumor weight, and number and distribution of nodules was noted by a gynecologic oncologist blinded to the treatment groups.

3.3 siRNA delivery to tumors

Gene silencing was done using specific sequences incorporated into DOPC nanoparticles as previously published [157, 158]. In short, siRNA is mixed with DOPC in the presence of excess tertiary butanol at a ratio of 1:10 (w/w) siRNA/DOPC. Tween20 is used as surfactant in the mixture, and the resulting product is vortexed, frozen in acetone/dry ice bath and lyophilized. Particles are hydrated with 0.9% saline before in vivo administration. Figure 20 shows delivery of siRNA at the tumor site.

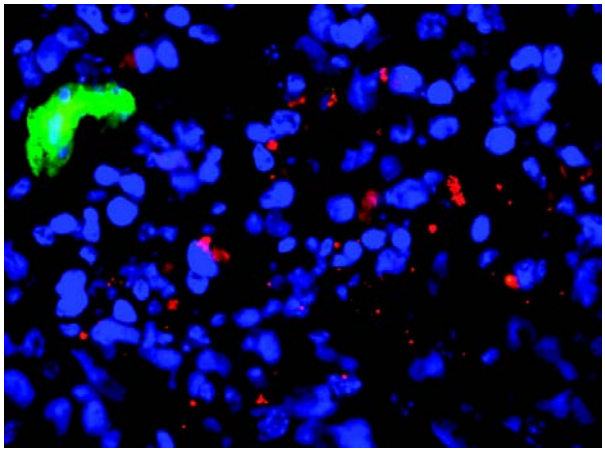

Figure 20: Tumors after delivery of siRNA in DOPC show siRNA in tumor, not clustered near the vasculature (CD31 in green) [158]. (C.N. Landen, A. Chavez-Reyes, C. Bucana, R. Schmandt, M.T. Deavers, G. Lopez-Berestein, A.K. Sood, Therapeutic EphA2 gene targeting in vivo using neutral liposomal small interfering RNA delivery, Cancer Res, 65 (2005) 6910-6918.) Cancer research : the official organ of the American Association for Cancer Research, Inc. by INTERNATIONAL CANCER RESEARCH FOUNDATION ; WILLIAM H.

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3.4 Immunofluorescence

Frozen slide-mounted tumor sections were fixed in acetone and washed in PBS. Slides were incubated with the following primary antibodies overnight: α-SMA (1:100, Abcam, Cambridge, MA, USA), FAP (1:100, R&D Systems, Minneapolis, MN, USA), GFP (1:500, Abcam), and RFP (1:100, Abcam). AlexaFluor488 and AlexaFluor594 fluorochromes (Jackson ImmunoResearch, West Grove, PA, USA) conjugated to secondary antibodies were used for primary antibody detection. Nuclei were identified by Hoechst stain (Invitrogen, Carlsbad, CA, USA).

3.5 Immunohistochemistry

Both frozen and paraffin sections were used for immunohistochemical analyses. Paraffin sections were heated for 20 min at 56°C and were deparaffinized in xylene and dehydrated-rehydrated in decreasing grades of alcohol and PBS. Antigen retrieval was performed in citrate buffer (pH 6.0) in the steamer for 30 min. Frozen sections were fixed in acetone and acetone-chloroform. After endogenous peroxide blocking with hydrogen peroxide in methanol and three washes with PBS, the slides were incubated with primary antibodies α-SMA (1:100, Abcam), FAP (1:100, R&D Systems), or Inhibin, Beta A (1:100, Abcam) overnight at 4°C. Sections were exposed to matching secondary antibodies

(Jackson ImmunoResearch) for 1 h at room temperature and staining was developed using 3,3'-diaminobenzidine. Nuclei were stained with hematoxylin.

3.6 Migration assay

Migration of NOF151 were measured *in vitro.* Inserts (8 µm; Millipore, Billerica, MA, USA) were coated with 1% gelatin for migration assays. A total of 50,000 cells were placed in each upper well and allowed to move toward conditioned medium in the lower chamber in the presence or absence of NE. Migration was assessed 6 h after treatment by fixing the cells in Protocol Hema3 (Thermo Fisher Scientific, Waltham, MA, USA). Cells were counted in randomly chosen high-power fields, and cell counts are reported as average numbers of cells migrated.

3.7 Collagen staining

Collagen distribution and density were assessed by using Masson trichrome staining (Abcam) for both paraffin and frozen slides. Paraffin sections were deparaffinized and dehydrated-rehydrated in alcohols and distilled water, and frozen slides were dried at room temperature followed by fixation in formalin for 30 min. Slides were then fixed in preheated Bouin Solution (Sigma-Aldrich). Slides were washed in tap water until the water ran clear, and Abcam's staining protocol was followed. Nuclei were stained with Weigert iron hematoxylin. Sirius Red/Fast Green Collagen staining for collagens 1 and 3 was done by using a kit (Chondrex, Redmond, WA, USA).

3.8 qRT–PCR

Total RNA was extracted from cells by using the Zymo Research RNA isolation kit with TRIzol reagent (Invitrogen) according to the manufacturer's standard protocol. Complementary DNA was synthesized from 1 µg of total RNA using the Verso cDNA Synthesis kit (Thermo Fisher Scientific) according to the supplier protocol, using random hexamers and oligo-dT primers in a 3:1 ratio. Quantitative PCR was performed using SYBR Green Master Mix on the 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) and standard protocols. The primer sequences used are included in tables 5 and 6.

B-actin	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG
ACTA2	CCAGAGCCATTGTCACACAC	CAGCCAAGCACTGTCAGG
INHBA	ATCTCGAAGTGCAGCGTCTT	GGAGGGCAGAAATGAATGAA
S100A4	TGTTGCTGTCCAAGTTGCTC	AACTAAAGGAGCTGCTGACCC
FAP	TCAGTGTGAGTGCTCTCATTGTAT	GCTGTGCTTGCCTTATTGGT
ADRB2	TCCACCTGGCTAAGGTTCTG	TGTCCTTCTACGTTCCCCTG
ACVR2a	GAAAGCCCAGTTGCTTAACG	GAAAGCCCAGTTGCTTAACG
ACVR2b	TGAGTACATGCTGCCCTTTG	TAATGGTGGGCCTCATCTTC
COL3A1	GATGGGGTCAAATGAAGGTG	GTGTGTTTCGTGCAACCATC
COL5A1	AGGATTTCCTGGACCAAAGG	TCTTGCCTTGGAAACCAGTC
COL5A2	CGGTGAAGAAGGCAAAAGAG	TTCTCCTTGAGCACCCTTTG

COL11A1	TCCTGGTGAAAAAGGACCAC	TTCTTTCCCAGGATGACCAG
CREB pos1	ACAACCCTTCACCGTTCTTG	ATAGGGGTAAAGCTGGTCAGG
CREB neg	GAGATTCTAAAGACCTGGGAAGG	CGACCCCAACCAACTTACAC

*Table 5: Human Sequences (*ChIP primers)*

B-actin	GCTACAGCTTCACCACCACA	TCTCCAGGGAGGAAGAGGAT
ACTA2	GTTCAGTGGTGCCTCTGTCA	ACTGGGACGACATGGAAAAG
S100A4	TTTGTGGAAGGTGGACACAA	CAGCACTTCCTCTCTCTTGG
FAP	CTTTGTGTTTCCTTCAGGTTTG	CTTTGGAGTTACCACCCTGG
ACVR2a	GGCGACATTGTTTTGCTACC	AGCCAACAACCTTGCTTCAC
ACVR2b	CTTTAAGCCCTTGCCTTTCC	TCACAGCCACAAAGTCGTTC
COL3A1	AGGATCTGTCCTTTGCGATG	TCTCCAAATGGGATCTCTGG
COL5A1	TGAACAGATGAAGCGACCAC	TATTCGCCATCTGGGAAGTC
COL5A2	TCCTCAGGGAATTGATGGAG	GCCATCTGAGCTGAAAAAGG
COL11A1	TGGTCATCCTGGGAAAGAAG	ACCCTTTTCGCCTTTAGAGC

Table 6: Murine Sequences

3.9 Proteome profiler

NOF151 cells were exposed to serum-free medium, medium conditioned by untreated cancer cells, or medium conditioned by NE-treated cancer cells. The

conditioned media were spun down at 1200 rpm to remove cell debris. Total protein from each culture was quantified according to Bradford protein assay, and 250 µg of each protein sample was assayed in parallel using a Human Cytokine Array kit (R&D Systems) according to the manufacturer's protocol.

3.10 Inhibin, Beta A ELISA

The Inhibin, Beta A ELISA kit was obtained from MyBiosource (San Diego, CA, USA). Supernatants from HeyA8 cells treated with NE or a specific beta-blocker, and with control or *INHBA* siRNA, were collected and spun down to remove cell debris. Supernatants were immediately stored at -80°C until use. The ELISA kit was used per the manufacturer's instructions. In short, a plate was incubated with standards or 1:10 diluted supernatants for 2 h. The plate was then incubated with biotin-labeled antibody for 1 h. After the plate was washed with PBS, it was developed with colorimetric reagent and read at 540nm. Results are expressed as pg protein/million cells.

3.11 Clinical samples

3.11.1 Renal cell carcinoma

Single cell suspension of patient tumor samples snap frozen at time of tissue collection was obtained from the Department Behavioral Science at MD Anderson Cancer Center. The protocols for handling and analyzing the specimens were approved by the MD Anderson Institutional Review Board. Patients provided written informed consent for the collection of specimens and clinical data in accordance with the ethics guidelines of and with approval from

the MD Anderson Institutional Review Board [86]. Patients were also completed several psychosocial questionnaires. The Centers for Epidemiologic Studies - Depression was used to assess depressive symptoms, with scores of 16 or above classified as meeting screening criteria for depressive symptoms with further evaluation recommended. 9 patients with stage 2 or 3 patients with 2 females and 2 males in low CESD score (CESD<10) and 3 males and 2 females with high CESD score (CESD Score>20) were chosen for analysis.

3.11.2 Ovarian Cancer

Gene expression array results from GSE9116 were used to assess stromal content in primary ovarian cancer patients with known depression scores. For this dataset, tumor samples and psychosocial and behavioral data was obtained from patients undergoing surgical resection of ovarian carcinoma. The protocols were approved by Institutional Review Boards at University of Iowa, University of Miami, and University of California Los Angeles. In short, 0.1 g of tumor was processed for total RNA, and genome-wide transcription analyzed using Affymetrix U133A high density oligonucleotide arrays.

3.11.3 Ovarian Cancer survival data using KMPlot

Primary ovarian cancer samples from The Cancer Genome Atlas (TCGA) was used to survival graphs using an online tool[159]. In short, this tool uses gene expression data (Affymetrix platform) from 522 patients to generate overall and progression-free survival using the probe 210511_s_at for *INHBA.* The samples were split by the median value and best cutoff was selected automatically. All stages, histology, TP53 and debulking statuses were included in the analysis.

3.12 Bioinformatics analysis

3.12.1 Netwalker

NetWalker was used to analyze whole tumor gene expression data from ovarian cancer patients with known CES-D scores. For this scale, scores of 14 or less are considered low stress and scores of 15 or 16 are considered high stress. Unbiased networks of upregulated and downregulated genes were developed by using Netwalker and Ingenuity Pathway Analysis. Analyses were run considering the ratio (average of High stress/low stress). The network with the highest scoring interactions throughout was identified, along with the functions of the genes involved.

3.12.2 Oncomine

To identify genes that are significantly correlated with *INHBA*, we analyzed the TCGA datasets for ovarian, colorectal, and breast cancers using Oncomine. Significantly co-expressed genes from ovarian, breast, and colorectal cancers were identified and tabulated.

3.12.3 RNA-Seq data

From the Level 3 (public) data from TCGA, including values from RNA-Seq assays, we generated plots that included summary values for *INHBA* from “RNA-SeqV2” across all tumor types, including all samples for which these data were available. The samples are ordered by tissue type.

3.12.4 Statistical analyses

MS Excel or GraphPad Prism software was used to analyze data. Continuous variables were compared by using the Student *t*-test or analysis of variance (ANOVA), and the Mann-Whitney test was used to compare differences. We determined that, using 2-way ANOVA, a sample size of 10 animals per group would provide an effective size of 1.3 with 80% power at a significance of $p=0.05$. We considered $p<0.05$ to be significant. All statistical analysis results were expressed as mean ± standard error of the mean.

4 Results

4.1 Stromal signature in ovarian tumors from patients with chronic adrenergic activation

My first goal was to identify potential pathways affected by adrenergic signaling. We accomplished this by first comparing high-grade serous cancers (HGSC) primary tumors from patients who had a high score on the CES-D from patients who had a low CES-D score and low tumoral NE levels. CES-D is a widely used self-report questionnaire to study depression in populations as this test has very high internal consistency and adequate retest reliability. These data were collected from a prospective cohort study (GEO GSE9116) [85] and we selected for genes that were significantly upregulated in the tumors of patients with high depressive symptoms (by at least 2-fold at the $p<0.05$ level). With the genes, we then performed an unbiased analysis of gene expression networks using NetWalker. I uncovered a strong signature indicative of a reactive stroma in tumors from patients with high depressive symptoms (Figure 24a and Table 7). I then compared the top 200 genes from these tumors with gene expression data comparing microdissected CAFs and normal fibroblasts (GEO GSE40643), and found 22 genes that were common to the high depression score group and the CAFs, indicating that CAFs are indeed enriched during chronic adrenergic signaling (Figure 21a and Table 8).

CAFs are associated with worse overall survival in ovarian cancer with documented roles in inflammation, immune cell infiltration and metastasis. To validate the biological relevance of these findings, I used beta adrenergic receptor (ADRB) positive cell lines in orthotopic mouse models of both human

(Skov3-ip1 and HeyA8) and murine (ID8-ip1) epithelial ovarian cancer. These tumor models show increased tumor growth, metastasis and angiogenesis under restraint stress. I induced chronic stress by using a well-characterized physical-restraint system in which animals are physically confined in a small chamber for 2 hours every day for 21 days [101, 109]. In addition, restraint stress causes sustained elevations of NE in tumors [109]. In tumors obtained at necropsy, samples from mice exposed to chronic restraint stress had significantly greater numbers of intratumoral cells positive for the CAF marker, alpha smooth muscle actin (α-SMA), in all models (Skov3-ip1: 4.23-fold increase, $p<0.05$; HeyA8: 1.88-fold increase, $p<0.01$; ID8-ip1 2.9-fold increase, $p<0.05$) (Figure 21b) [134]. Fibroblasts in tumors can also flank big vessels, and hence smooth muscle cells can also stain positive for α-SMA, we also co-stained for CD31 and α-SMA. The significantly higher α-SMA–positive signal in tumors from mice that underwent chronic stress than in controls was independent of CD31-positive cells, indicating that restraint stress induced an increase in the CAF phenotype (CD31-independent a-SMA+ cells: 1.8-fold increase) (Figure 21c) [135, 160]. There was a time-dependent CAF phenotype which was more pronounced in mice stressed for 21 days than in those stressed for 7 days (Skov3-ip1: 2.1-fold increase, $p<0.05$; ID8-ip1 2.2-fold increase, $p<0.05$) (Figure 24d). In addition to α-SMA, other CAF markers such as fibroblast-activated protein (FAP), Platelet Derived Growth Factor Receptor-α and S100A4 are commonly used. I also evaluated another CAF marker, FAP and found a similar increase in its expression under restraint stress in Skov3-ip1 model (Figure 22b). CAFs are an integral part of

tumor microenvironment and CAFs were detected in primary as well as all metastatic sites in these preclinical models of ovarian cancer. The CAF content was increased by restraint stress compared to controls in the Skov3 model (2-fold increase, $p<0.05$) in primary site and common places of ovarian cancer metastasis such as omentum, peritoneum, and mesentery (Figure 22c). All of this data shows adrenergic signaling can accelerate CAF induction within ovarian cancer tumors in patient and preclinical models.

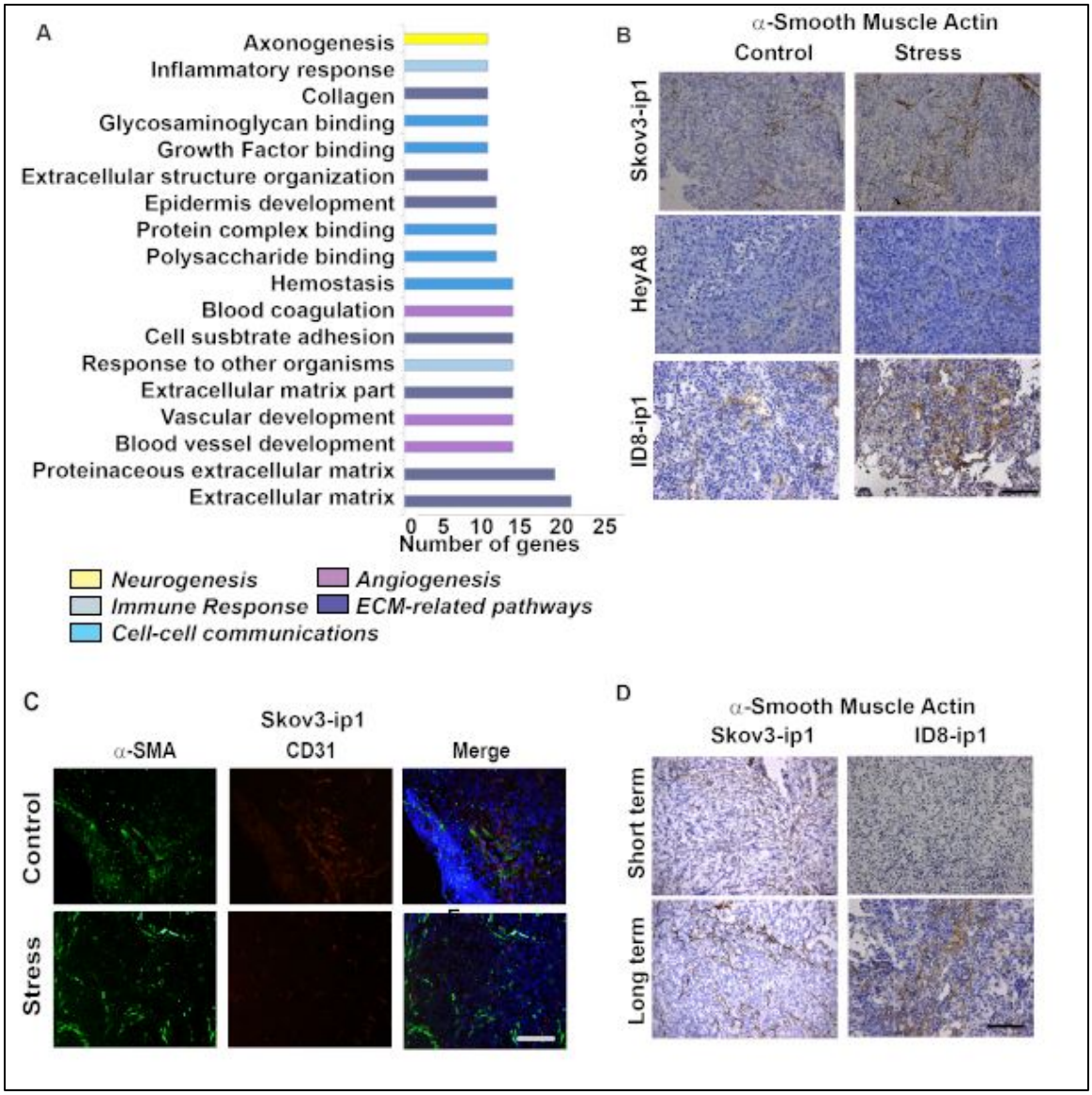
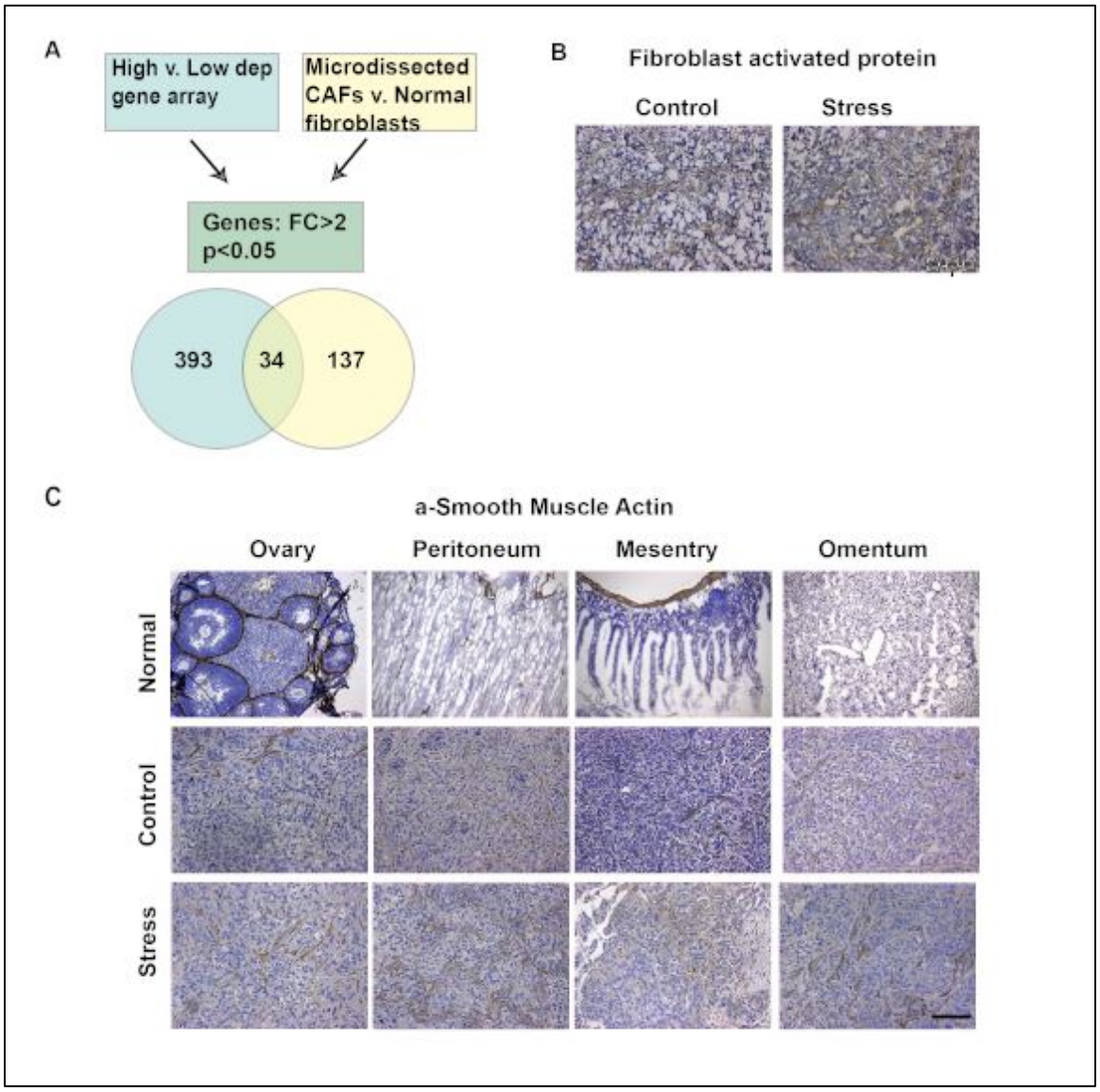


Figure 21: Chronic stress accelerates induction of the cancer-associated fibroblast (CAF) phenotype in ovarian carcinoma

(a) Results from NetWalker analysis to identify networks of upregulated genes in ovarian cancer patients with high CES-D (i.e., depression) scores. (b) Expression of alpha–smooth muscle actin (α-SMA, a CAF marker) in micrographs of representative tumors from control and restraint-stressed mice in adrenergic

receptor–positive Skov3-ip1, HeyA8, and ID8-ip1 models. (c) Expression of α-SMA and CD31 (blood vessel marker) in micrographs of representative Skov3-ip1 tumors from control and stressed mice. (d) Expression of α-SMA in micrographs of representative tumors from mice subjected to short-term (7 days) or long-term (21 days) stress. Scale bars, 100μM, n=5/group for all data.

Figure 22: Restraint stress increases CAF content in primary as well as metastatic sites.



(a) Venn diagram showing comparison of genes that are upregulated 2-fold in tumor samples from ovarian cancer patients with a high depression (dep) score compared to those with a low depression score and in microdissected cancer-associated fibroblasts (CAFs) compared to normal fibroblasts in primary ovarian cancer. FC, fold-change. (b) Expression of CAF marker fibroblast activated

protein (FAP) in micrographs of representative tumors from control and restraint-stressed mice in the adrenergic-receptor positive Skov3-ip1 model. (c) Expression of CAF marker alpha–smooth muscle actin (α-SMA) in micrographs of representative normal tissues and matched metastatic tumors from the Skov3-ip1 mouse model. Scale bars, 100μM, n=5/group for FAP data, n=3/group for metastatic data.

Annotation ID	Functional Annotation	Genes
GO:0031012	extracellular matrix	COL5A3, COL5A1, COL1A2, LGALS1, COL1A1, MMP11, PI3, SPARC, THBS1, COL8A1, COL8A2, CTGF, TIMP2, TNC, DCN, COL6A1, COL3A1, COL17A1, VWF, CTSD
GO:0005578	proteinaceous extracellular matrix	COL5A3, COL5A1, COL1A2, LGALS1, COL1A1, MMP11, PI3, SPARC, COL8A1, COL8A2, CTGF, TIMP2, TNC, DCN, COL6A1, COL3A1, COL17A1, VWF
GO:0001568	blood vessel development	COL5A1, COL1A2, COL1A1, ITGA5, THBS1, ANPEP, COL8A1, COL8A2, C5AR1, CTGF, TGFBR2, COL3A1, LYL1
GO:0001944	vasculature development	COL5A1, COL1A2, COL1A1, ITGA5, THBS1, ANPEP, COL8A1, COL8A2, C5AR1, CTGF, TGFBR2, COL3A1, LYL1
GO:0044420	extracellular matrix part	COL5A3, COL5A1, COL1A2, COL1A1, SPARC, COL8A1, COL8A2, TIMP2, TNC, DCN, COL6A1, COL3A1, COL17A1
GO:0051707	response to other organism	CCL8, ACTA2, CD14, LBP, PLA2G2A, GPX3, SOD2, CCL5, C5AR1, CXCL12, HCK, DCN, NFKBIA

GO:0031589	cell-substrate adhesion	COL5A3, LGALS1, ITGA11, COL1A1, ITGB5, ITGA3, THBS1, ITGB2, COL8A1, CTGF, COL3A1, COL17A1, VWF
GO:0007596	blood coagulation	COL1A2, COL1A1, RAC2, ITGA5, ITGA3, SPARC, THBS1, ITGB2, TFPI, SLC16A3, PECAM1, COL3A1, VWF
GO:0007599	hemostasis	COL1A2, COL1A1, RAC2, ITGA5, ITGA3, SPARC, THBS1, ITGB2, TFPI, SLC16A3, PECAM1, COL3A1, VWF
GO:0030247	polysaccharide binding	COL5A3, COL5A1, SUSD2, CCL8, THBS1, CD14, ABP1, CTGF, TGFBR2, NCAN, DCN
GO:0032403	protein complex binding	COL5A1, ITGB5, ITGA5, THBS1, CTGF, TIMP2, HCLS1, COL3A1, VWF, NFKBIA, CTSB
GO:0008544	epidermis development	COL5A3, COL5A1, COL1A2, COL1A1, LTB, EVPL, KRT17, PPL, CTGF, COL3A1, COL17A1
GO:0043062	extracellular structure organization	COL5A3, COL5A1, COL1A2, COL1A1, MYH11, COL8A2, CTGF, TNC, NCAN, COL3A1
GO:0019838	growth factor binding	COL5A1, COL1A2, COL1A1, IGFBP3, THBS1, IGFBP4, CTGF, TGFBR2, COL6A1, COL3A1
GO:0005539	glycosaminoglycan binding	COL5A3, COL5A1, CCL8, THBS1, CD14, ABP1, CTGF, TGFBR2, NCAN, DCN

GO:0005581	collagen	COL5A3, COL5A1, COL1A2, COL1A1, COL8A1, COL8A2, DCN, COL6A1, COL3A1, COL17A1
GO:0006954	inflammatory response	ALOX5AP, ALOX5, CCL8, THBS1, IGFBP4, CD14, ITGB2, LBP, PLA2G2A, CCL5
GO:0007409	axonogenesis	COL5A1, COL1A2, COL1A1, RAC2, ITGA5, MYH11, UCHL1, NCAN, COL6A1, COL3A1

Table 7: Top networks upregulated in tumors from patients with high CES-D scores compared to those with low CES-D scores, with corresponding involved genes

Affymetrix	Symbol	Patient Samples	Gene array for CAFs
202207_at	ARL4C	3.05	3.03
212077_at	CALD1	2.12	2.27
37892_at	COL11A1	4.47	5.674
201852_x_at	COL3A1	2.15	4.399
212489_at	COL5A1	2.09	3.809
221730_at	COL5A2	2.33	4.267
221541_at	CRISPLD2	3.11	3.083
201360_at	CST3	2.04	2.065
209101_at	CTGF	4.52	2.773
213274_s_at	CTSB	2.12	2.691
210764_s_at	CYR61	2.34	2.305
219454_at	EGFL6	2.71	3.673

211719_x_at	FN1	2.28	2.777
211911_x_at	HLA-B	2.15	2.27
211990_at	HLA-DPA1	2.35	2.561
209312_x_at	HLA-DRB1	2.62	2.445
211634_x_at	IGHM	2.12	2.326
216560_x_at	IGLC1	2.73	3.048
210511_s_at	INHBA	2.14	3.482
203417_at	MFAP2	2.08	2.52
209596_at	MXRA5	2.94	4.471
201058_s_at	MYL9	2.21	2.151
202620_s_at	PLOD2	2.14	2.562
210809_s_at	POSTN	2.88	4.933
218723_s_at	RGCC	2.03	2.767
203889_at	SCG5	4.31	2.185
212667_at	SPARC	2.07	2.984
203083_at	THBS2	2.09	2.568
201147_s_at	TIMP3	2.23	2.324
215034_s_at	TM4SF1	2.87	2.239
202241_at	TRIB1	2.43	2.374
203868_s_at	VCAM1	2.45	2.108
211571_s_at	VCAN	2.55	4.584

Table 8: Genes common to GSE9116 and GDS40643, arranged alphabetically

4.2 Promotion of CAF phenotype by chronic adrenergic signaling

There are 3 ADRB receptors with distinct expression patterns in tissue and varied biological functions. I first wanted to identify if this was due to direct effect of NE signaling in fibroblasts and to do this, I used the A2780 cells that are negative for all ADRB receptors. In this model, restraint stress has no effect on tumor growth or metastasis but if there are direct effects of NE signaling on fibroblasts, I hypothesized that there would be an increase in CAF content. There was no significant difference in α-SMA expression between tumors from control and restraint-stress groups (A2780: 1.1-fold change, $p>0.05$) (Figure 23a). The increase in CAF content during restraint stress was only seen in ADRB+ cells, indicating the important role of adrenergic signaling in tumor cells for driving the stress-induced CAF phenotype.

Catecholamines such as norepinephrine, epinephrine can trigger signaling in tumor and other cell types with ADRB and ADRA receptors respectively [88]. Compared to epinephrine, most tumor effects are driven by norepinephrine and in patient tumors; depressive symptoms are associated with NE and not epinephrine. To further assess the roles of norepinephrine signaling as the primary catecholaminergic driver of the CAF phenotype under chronic stress, I assessed the expression of α-SMA after chronic restraint stress in tumors from adrenalectomized mice that had been injected with ID8-ip1 cells. In these animals, the adrenal glands were surgically removed and tumor cells were injected after the animal recovered. By adrenalectomy, we are eliminating the primary source of circulating epinephrine and norepinephrine which are adrenal

glands, but preserving the primary source of norepinephrine (which is released predominately from local peripheral nerves) [161]. The tumors from adrenalectomized mice had significant increases in α-SMA levels under restraint stress (2.5- and 3.1-fold increases over controls in sham surgery and adrenalectomy groups, respectively; $p<0.05$) (Figure 23b), implying a primary role of NE. To further define the role of adrenergic receptors downstream of adrenergic signaling, I used beta-blockers and agonists. Propranolol is an FDA-approved beta-blocker for cancer and cardiovascular ailments that works by blocking all 3 ADRB receptors. Isoproterenol and terbutaline are broad and ADRB2-specific beta-agonists which can be given systemically to activate ADRB receptors. I first started by blocking all 3 ADRB using propranolol. I treated tumor bearing mice with PBS or propranolol, a nonspecific beta-blocker that blocks all downstream beta-adrenergic signaling in cells. Propranolol abrogated the stress-mediated increases in α-SMA level in the HeyA8 model (PBS: 2.2-fold increases; $p<0.05$, propranolol: 0.87-fold change, $p>0.05$) (Figure 23c) and reduced gene expression of CAF markers in the Skov3-ip1 model (Figure 23c). Conversely, treatment of tumor bearing mice with either isoproterenol (non-specific beta agonist) or terbutaline (ADRB2-specific agonist) increased CAFs by 2.5 and 3.1-fold, respectively in tumors (Figure 23d). Since agonists and beta-blockers are not specific to tumor cells, and I wanted to see specifically the contribution of adrenergic-signaling in tumor cells, we used siRNA incorporated into DOPC nanoliposomes to silence ADRB2 in tumor cells. siRNA against human-specific ADRB2 was incorporated into DOPC nanoliposomes and given at 5 ug per

mouse biweekly by intraperitoneal injections. In the HeyA8 mouse model in which ADRB2 was silenced by siRNA incorporated into nanoliposomes, stress-mediated increases in α-SMA expression were abrogated (Figure 23e).

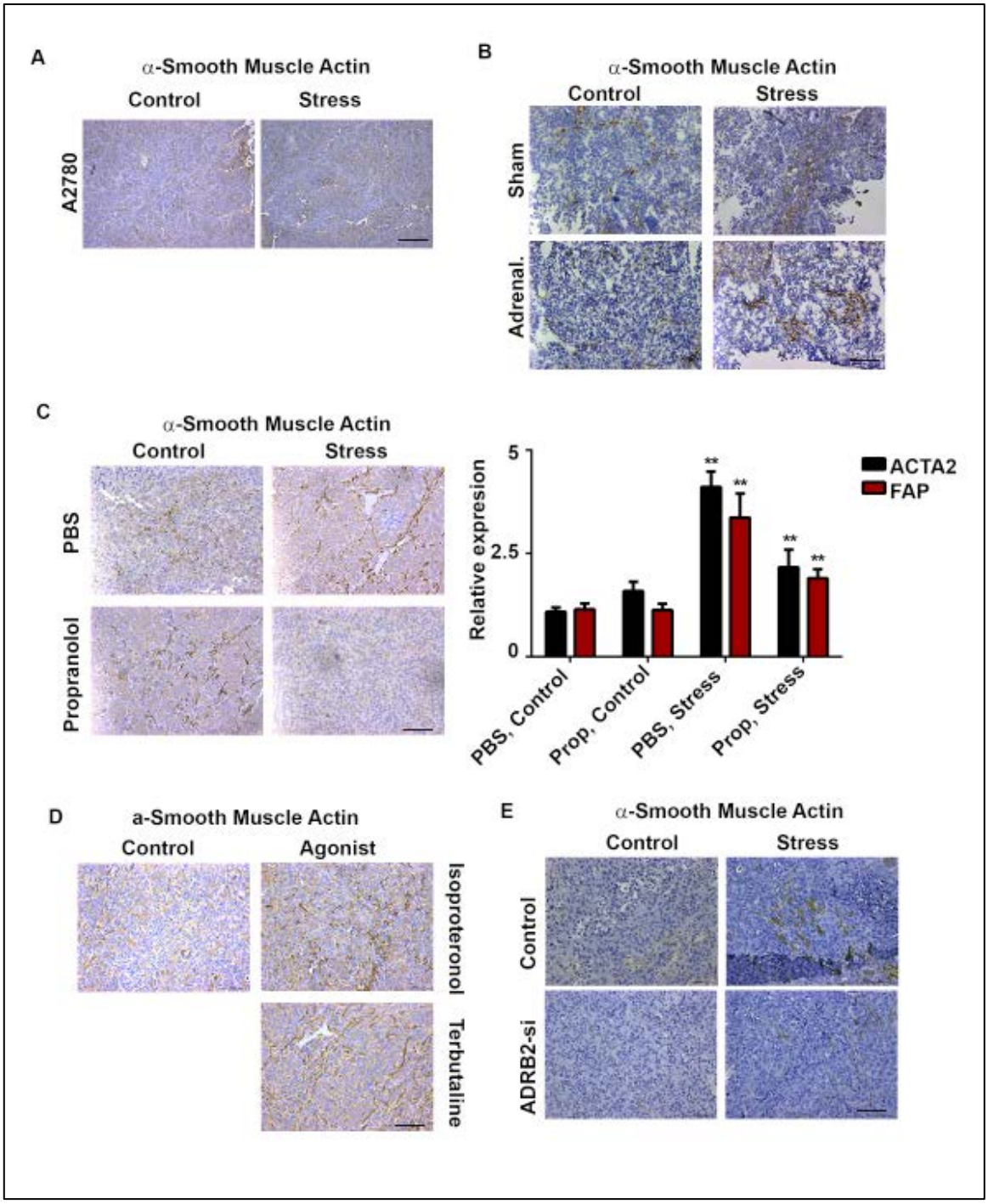


Figure 23: Induction of the cancer-associated fibroblast (CAF) phenotype in ovarian carcinoma is due to the indirect effects of adrenergic signaling.

(a) Expression of CAF marker alpha–smooth muscle actin (α-SMA) in micrographs of representative tumors from control and restraint-stressed mice in the adrenergic-receptor (ADRB)–negative A2780 (orthotopic) model. (b) Expression of α-SMA in micrographs of representative ID8 tumors from control and stressed mice that underwent adrenalectomy (Adrenal.) or sham surgery. (c) Expression of α-SMA in micrographs of representative HeyA8 tumors from control and stressed mice treated with the nonspecific beta-blocker propranolol or phosphate-buffered saline solution (PBS, controls). (d)) Expression of α-SMA in micrographs of representative HeyA8 tumors from control and stressed mice treated with PBS (control), nonspecific beta-agonist isoproterenol, or ADRB2-specific agonist terbutaline. € Expression of α-SMA in micrographs of representative HeyA8 tumors from control and stressed mice treated with control or ADRB2 siRNA loaded into nanoliposomal DOPC. Scale bars, 100μm, n=5/group for all data.

We also examined the consequences of tumor cell adrenergic stimulation for fibroblast activation *in vitro*. We used NOF151, a normal fibroblast cell line that is derived from normal ovary and has been previously characterized [139]. Similar to fibroblasts in preclinical models, these cells have low expression of adrenergic receptors and therefore do not respond to direct NE treatment, as indicated by lack of increase in cAMP levels (Figures 24a and 24b). However NOF151 cells exposed to either control medium conditioned by untreated cancer cells, or medium conditioned by NE-treated cancer cells, we saw increased expression of CAF markers *ACTA2, FAP,* and *S100A4* RNA and α-SMA protein (Figures 24c and 24d). Two characteristics of transformed fibroblasts *in vitro* are increased production of pro-inflammatory cytokines and increased migration [141, 142]. Conditioned medium from NE-treated cancer cells increased expression of pro-inflammatory cytokines such as MCP1, CXCL1, MIF, GM-CSF, and G-CSF (Figure 24e) and migratory potential **(**Figure 24f) in NOF151 cells. Taken together, these results demonstrate that NE exposure induces tumor cells to express soluble factors that enhance activation of normal fibroblasts.

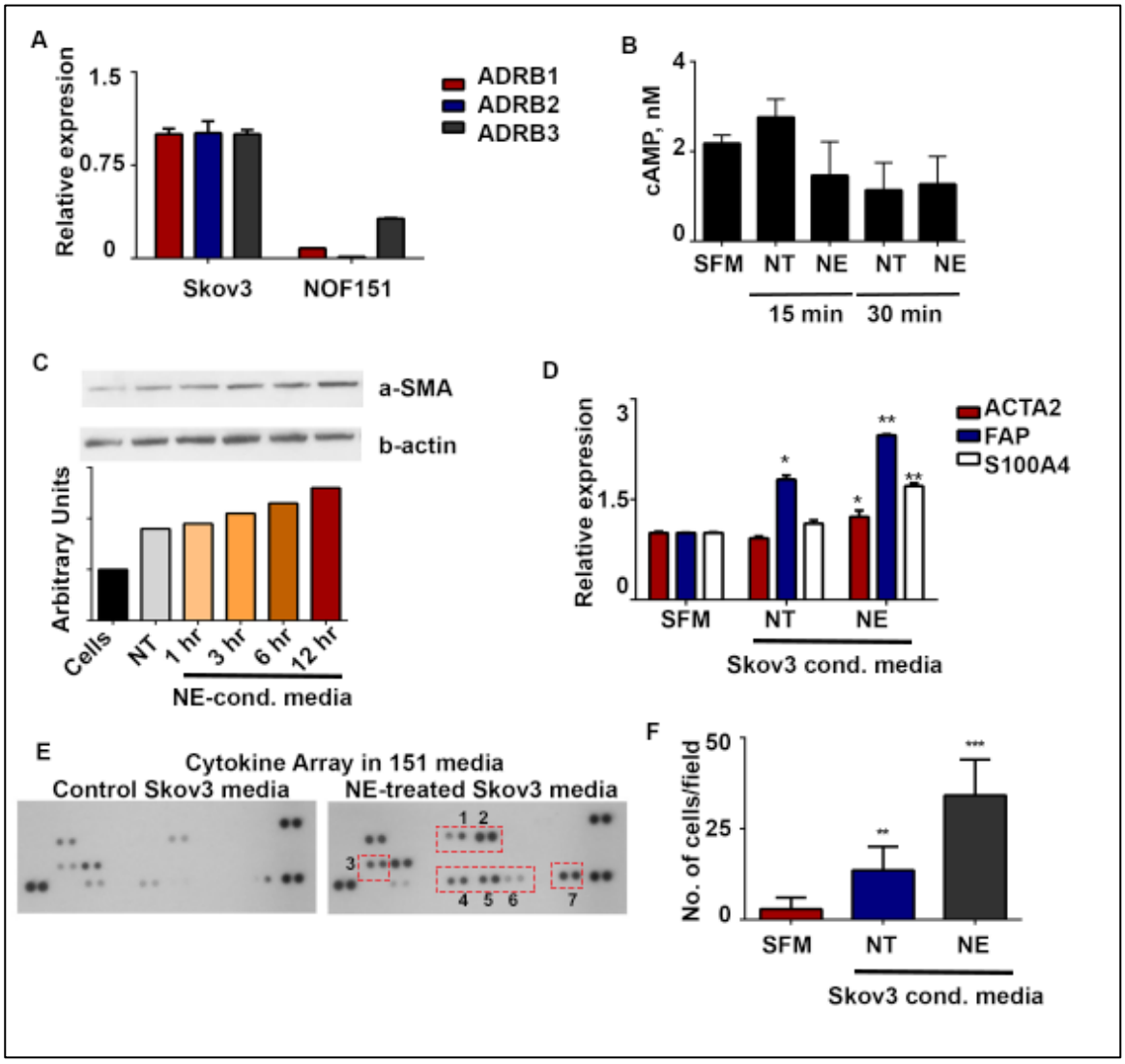
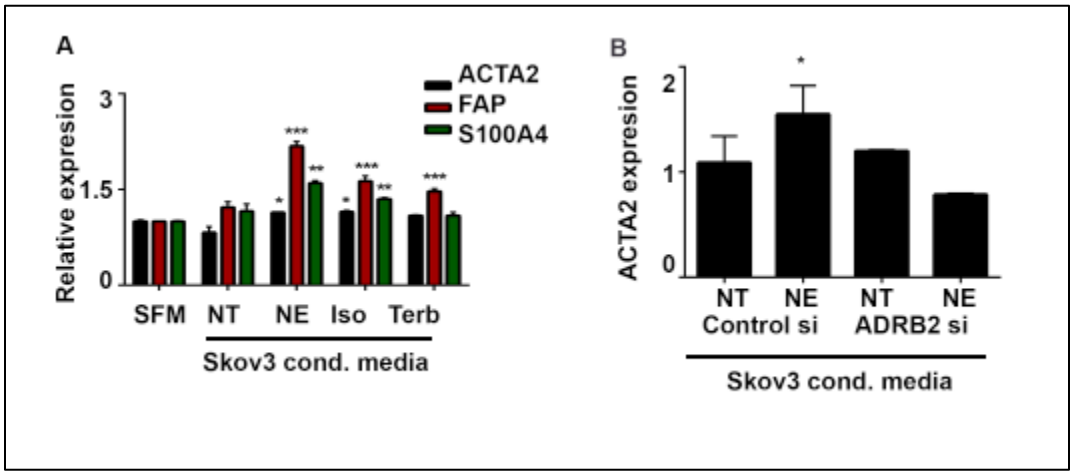


Figure 24: Conditioned media from NE-treated cancer cells accelerate transformation of normal ovarian fibroblasts

(a) Expression of beta-adrenergic receptors (ADRB) in NOF151 normal

fibroblasts relative to ADRB-positive Skov3-ip1 cells. (b) ELISA for cAMP in NOF151 treated with norepinephrine (NE) for 15 or 30 min or not treated (NT). (c) Expression of CAF marker alpha–smooth muscle actin (-SMA) in NOF151 cells after exposure to medium conditioned by NE-treated Skov3-ip1 cells. (d) Expression of CAF markers *ACTA2, S100A4,* and *FAP* in NOF151 cells after exposure to medium conditioned by Skov3-ip1 cells treated with NE or NT. (e) Immunoblot multiplex assay for pro-inflammatory cytokines in NOF151 cells exposed to medium conditioned by NE-treated or NT Skov3-ip1 cells. (1: GCSF, 2: GM-CSF, 3:IL1a, 4: CCL2, 5: MIF, 6: CCL3, 7: PAI1) (f) *In vitro* migratory potential of NOF151 cells toward serum-free medium (SFM), medium conditioned by untreated Skov3-ip1 cells, or medium conditioned by NE-treated SKov3-ip1 cells through 0.1% gelatin over 6 h was assessed by fixing and counting the number of cells migrated per high-power field. Scale bars, 100µM. Statistical significance was obtained using the 1-way ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p<0.001$.

Consistent with the adrenergic receptor specificity documented above *in vivo*, NOF151 cells exposed *in vitro* to medium from isoproterenol- or terbutaline-treated cancer cells showed greater α-SMA induction than those exposed to medium conditioned by non-treated cells (Figure 25a). Conditioned medium from ADRB2-silenced cancer cells abrogated expression of CAF marker *ACTA2* in



NOF151 cells (Figure 25b).

Figure 25: Restraint stress increases α-SMA in an ADRB2-dependent manner

(a) Expression of CAF markers *ACTA2, S100A4,* and *FAP* in NOF151 cells exposed to serum-free medium (SFM) or to medium conditioned by Skov3-ip1 cells that were untreated (NT) or treated with norepinephrine (NE), nonspecific beta-agonist isoproterenol (Iso), or ADRB2-specific agonist terbutaline (Terb). (b) ACTA2 expression in NOF151 treated with ADRB2 or control siRNA and conditioned with NT or NE-treated Skov3-ip1 cells. Scale bar, 100 □m. Statistical significance was obtained using the 1-way ANOVA: *p < 0.05, **p < 0.01, ***p<0.001.

4.3 Stromal effects of chronic stress

I next wanted to study the biological consequences of fibroblast activation by chronic stress. To obtain an unbiased approach, I used NetWalker for gene expression analysis to identify upregulated networks in patient gene expression array from GSE9116 (Figure 26a). From this analysis and the results shown in Table 4, I identified that there was an increased collagen signature in tumors during chronic stress. Collagens in ovarian tumors have been shown to drive metastasis, impede immune cell infiltration and diminish chemotherapy efficacy. To assess collagen content in tumors, histological staining is primarily used including Masson's Trichrome and Sirius. Masson Trichrome is used to identify connective tissue from cells in histological samples. This is a chromogenic, chemical based assay in which nuclei are stained black, connective tissue blue and cytoplasm stains pink. Sirius is another chromogenic assay that preferentially stains collagens 1 and 3. Masson trichrome staining and Sirius staining of Skov3-ip1 and HeyA8 tumors confirmed elevated collagen deposition after restraint stress in the *in vivo* mouse model (increase of 2.1-fold, $p<0.05$) **(**Figure 26b**).** Stress-mediated collagen deposition in tumors was largely abrogated by propranolol treatment in both models (1.64- and 1.01-fold increases over controls in PBS- and propranolol-treated groups, respectively; $p=0.05$ for PBS group) **(**Figure 26c**).** Similarly, restraint stress–induced increases in collagen were also abrogated by ADRB2 silencing, as shown by both trichrome and Sirius staining (Figure 26d). Treating animals with the non-specific beta-agonist or the ADRB2-specific agonist resulted in significant increases in tumoral

collagen (1.81- and 1.75-fold increases for isoproterenol- and terbutaline-treated groups, respectively, over PBS-treated controls; $p<0.05$) **(**Figure 26e).

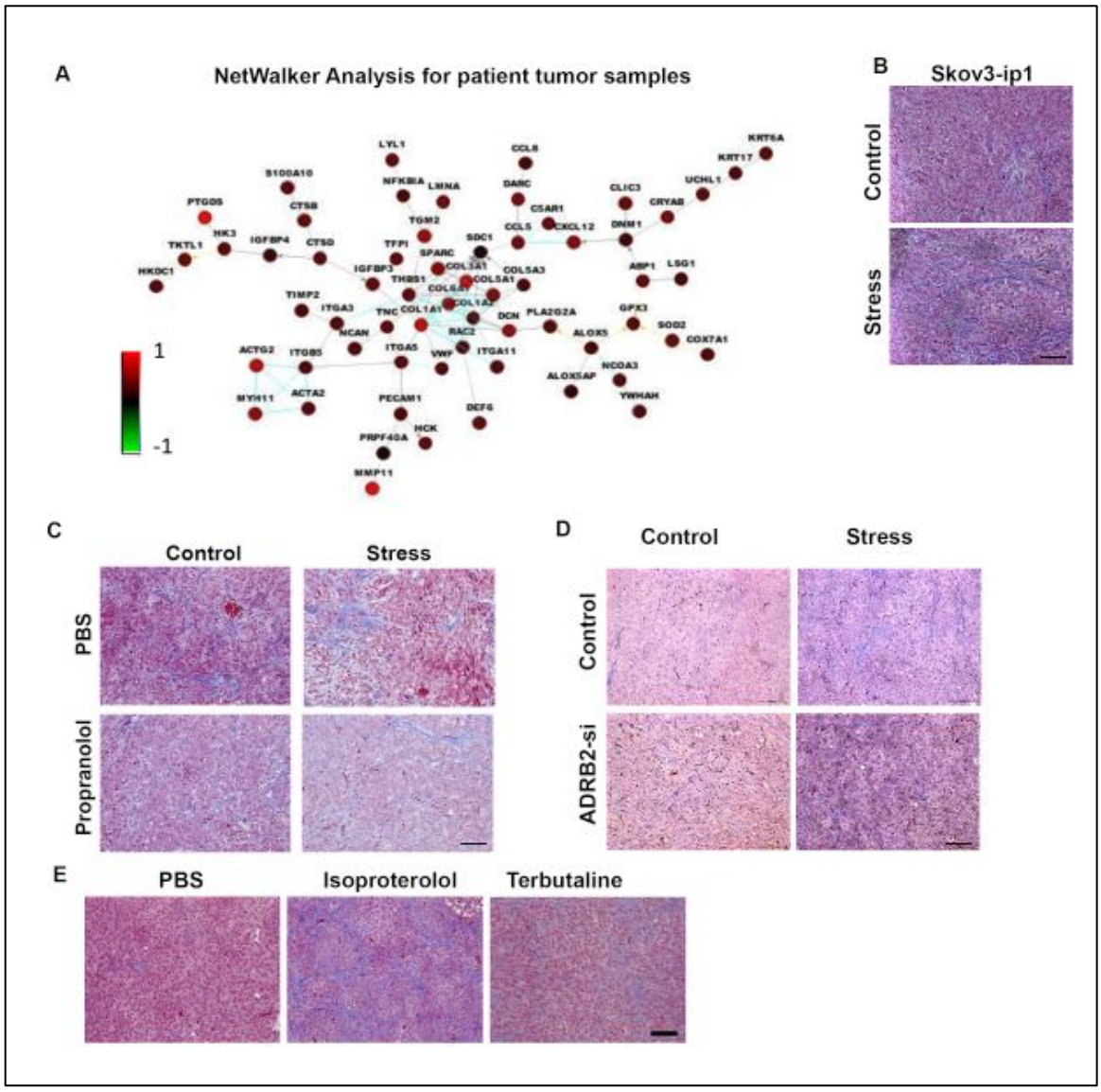


Figure 26: Adrenergic signaling increases collagen deposition and extracellular matrix formation in ovarian tumors.

(a) An unbiased network of genes differentially upregulated in tumor samples from high depression score and low depression score patients. (b) Expression of collagen identified by Masson trichrome staining in micrographs of representative Skov3-ip1 tumors from control and restraint-stressed mice. (c) Expression of collagen in representative Skov3-ip1 tumors from control and stressed mice treated with nonspecific beta-blocker propranolol or phosphate-buffered saline solution (PBS, controls). (d) Expression of collagen identified by Masson trichrome staining in micrographs of representative HeyA8 tumors from control and stressed mice treated with control or ADRB2 siRNA. (e) Expression of collagen identified by Masson trichrome staining in micrographs of representative HeyA8 tumors from control and stressed mice treated with PBS (control), nonspecific beta-agonist isoproterenol, or ADRB2-specific agonist terbutaline. (Scale bars, 100μM, n=5/group for all data.

Sirius stain is also a chromogenic stain more specific to collagens 1 and 3. Both of these collagens were identified to be elevated in chronic adrenergic signaling. Sirius staining showed collagens was significantly increased by 2-fold in both HeyA8 and Skov3 tumors under chronic restraint stress (figure 27a). Similar to Trichrome staining, Sirius stains showed adrenergic-mediated increases in collagens 1 and 2 were abrogated during systemic propranolol treatment (Figure 27b). I also assessed expression levels of several collagens identified from the patient datasets using murine-specific primers to identify only stromal-based changes. Several genes involved in collagen synthesis were elevated in tumors from mice exposed to restraint stress, and these increases were also abrogated by propranolol (Figure 27c). Silencing ADRB2 in tumor cells using DOPC nanoliposomes also decreased collagen 1 and 3 in tumors (Figure 27d). In vitro, NOF151 normal fibroblasts exposed to medium conditioned by NE-treated cancer cells showed similar elevations in the same collagen genes (Figure 27e). However, NOF151 conditioned to ADRB2 silenced Skov3 cells did not show these elevations in collagen genes (Figure 27f).

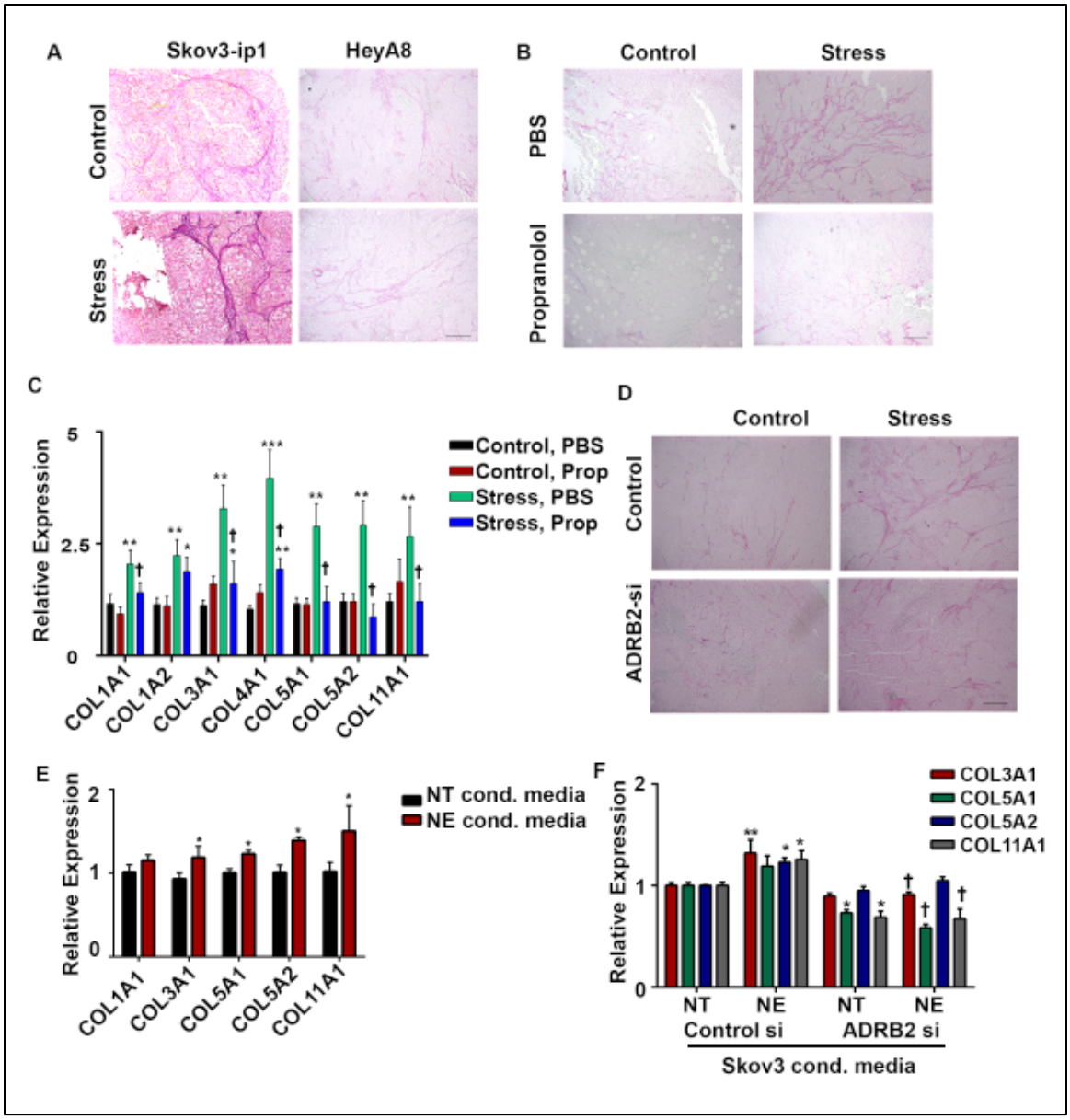


Figure 27: Restraint stress increases collagen levels in tumors in an ADRB2 dependent manner

(a) Expression of collagen detected by Sirius staining in micrographs of representative Skov3-ip1 and HeyA8 tumors from control and restraint-stressed mice. (b) Expression of collagen detected by Sirius staining on micrographs of representative HeyA8 tumors from control and stressed mice treated with

nonspecific beta-blocker propranolol or PBS. (c) Expression of collagen genes in Skov3-ip1 tumors from control and stressed mice treated with nonspecific beta-blocker propranolol (Prop) or Saline. (d) Expression of collagen detected by Sirius staining in micrographs of representative HeyA8 tumors from control and stressed mice treated with control or ADRB2 siRNA. (e) Expression of collagen genes in NOF151 cells exposed to medium conditioned by untreated (NT) or norepinephrine (NE)-treated Skov3-ip1 cells. Scale bars, 100μM. (f) Expression of collagen genes in NOF151 cells exposed to medium conditioned by untreated (NT) or norepinephrine (NE) and ADRB2 silenced Skov3 cells. Scale bars, 100μM. Statistical significance was obtained using the 1-way ANOVA: *$p < 0.05$, **$p < 0.01$ compared to controls, †$p<0.05$ compared to stress or NE-treated cells.

4.4 Induction of CAF phenotype by NE-mediated Inhibin, Beta A production

My data demonstrates that cell contact between cancer cells and fibroblasts are not required for the NE-induced CAF phenotype. I hypothesized that cytokines, growth factors or other such extracellular factors were central to this phenomenon. Using a gene array dataset of Skov3-ip1 and HeyA8 cells treated with NE, we identified several potential genes that are induced by NE that could be mediators of this effect [101]. I also wanted to identify potential upstream regulators that are central to CAF activation in ovarian tumors. For this, I used Ingenuity Pathway Analysis (IPA) on gene arrays of microdissected CAFs and normal ovarian tissues to identify upstream regulators of CAFs [139]. Combining the gene array and systems-based analyses, I identified *IL6* (interleukin 6) and *INBHA* (Inhibin, Beta A) as factors that could potentially drive CAF expression (Figure 28a) [101]**.** IL6 is an important proinflammatory cytokine that is potently induced upon NE treatment in ovarian cancer cells. In addition, elevated plasma levels of IL6 are also associated with ovarian cancer patients who show depressive symptoms. Inhibin, Beta A is also elevated in plasma of ovarian cancer patients. However, there is no knowledge of NE driving Inhibin, Beta A levels in cells. To identify which gene is more likely to be the mediator, I used coexpression analysis of genes using Oncomine. Oncomine is a web-based portal with publicly available datasets such as TCGA that enables users to do survival, expression and other analysis. I used Oncomine to analyze co-expression of *INHBA* and *IL6* with genes upregulated in ovarian cancer samples

included in The Cancer Genome Atlas (TCGA). *INHBA*, but not *IL6*, was highly correlated with stroma-related genes that were upregulated in both patient datasets (Tables 9 and 10**)**. Therefore, I focused primarily on *INHBA* for subsequent experiments.

In the GEO data set GSE9116, I noted *INHBA* mRNA levels were increased 2.14 fold in tumors from patients with high depression scores (Figure 28b**)**. In the *in vivo* preclinical models, restraint stress significantly increased Inhibin, Beta A levels in Skov3-ip1 and HeyA8 tumors (2.3- and 3.1-fold increases over non-stress controls, respectively, $p<0.05$) (Figure 28c). Treatment with propranolol abrogated the increases in tumoral *INHBA* indicating that blocking beta-adrenergic receptors can block this effect (Figure 28d).

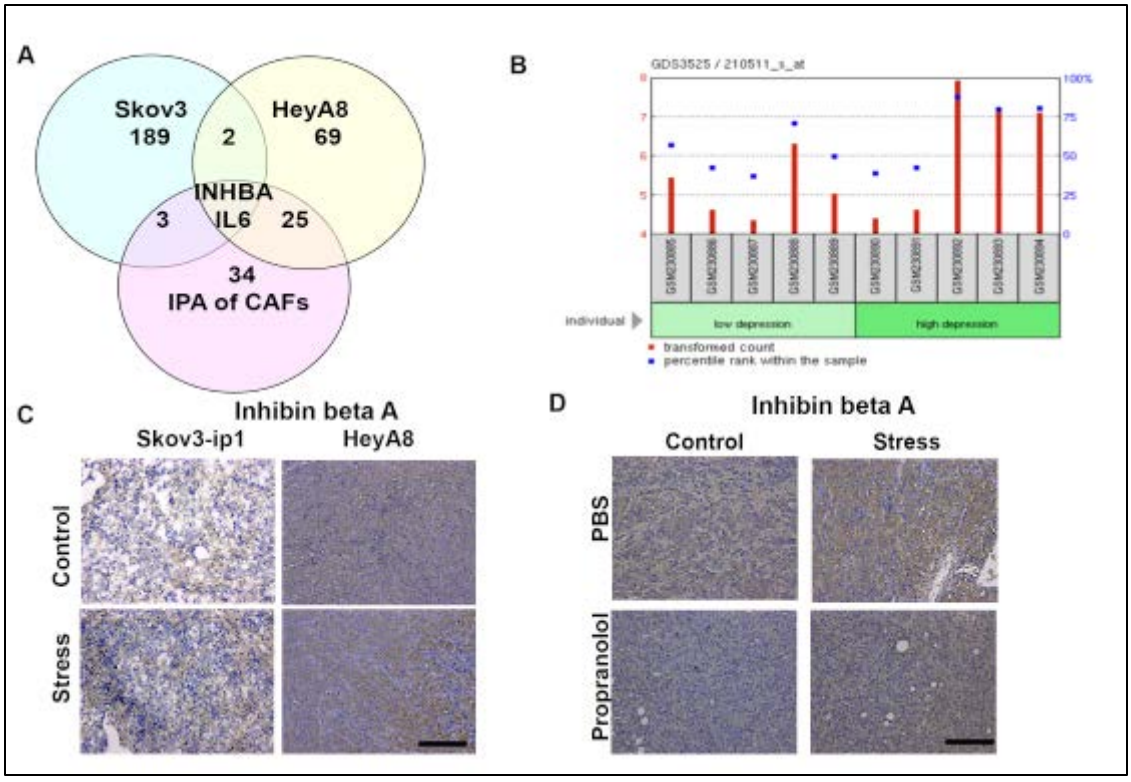


Figure 28: Induction of cancer-associated fibroblasts (CAF) in ovarian carcinoma is mediated by Inhibin, Beta A downstream of norepinephrine.

(a) Schema used for Ingenuity Pathway Analysis (IPA) of three different ovarian carcinoma cell lines to identify upstream regulators of CAF induction. (b) Validation of Inhibin, Beta A levels in tumors from patients with high depression scores and low depression scores; data were obtained from GEODATASET GSE9116. (c) Expression of Inhibin, Beta A in micrographs of representative Skov3-ip1 and HeyA8 tumors from control and restraint-stressed mice. (d) Expression of Inhibin, Beta A in micrographs of representative HeyA8 tumors from control and stressed mice treated with nonspecific beta-blocker propranolol or phosphate-buffered saline solution (PBS, control). Scale bars, 100μM, n=5/group for all data.

Gene Symbol	Reporter ID	Correlation
INHBA	210511_s_at	1
THBS2	203083_at	0.90183127
COL11A1	204320_at	0.8865286
COL11A1	37892_at	0.8865286
FAP	209955_s_at	0.8479447
CTSK	202450_s_at	0.8479447
VCAN	211571_s_at	0.8479447
SPARC	212667_at	0.8177246
AEBP1	201792_at	0.8177246
COL1A2	202403_s_at	0.8177246
COL6A3	201438_at	0.8177246
COL3A1	201852_x_at	0.8177246
COL5A1	212489_at	0.8177246
COL5A2	221730_at	0.8177246
MMP2	201069_at	0.81008625
SNAI2	213139_at	0.8011878
FBN1	202765_s_at	0.8011878
FN1	211719_x_at	0.7849791
COL10A1	217428_s_at	0.7731993
-	205941_s_at	0.7731993
CDH11	207172_s_at	0.7619725

DCN	209335_at	0.7619725
LUM	201744_s_at	0.7619725
SERPINF1	202283_at	0.731467
CRISPLD2	221541_at	0.72007775
ASPN	219087_at	0.72007775
POSTN	210809_s_at	0.72007775
TMEM158	213338_at	0.7155617
OLFML2B	213125_at	0.7155617
ADAM12	213790_at	0.7155617
ADAM12	202952_s_at	0.7155617
NTM	222020_s_at	0.7155617
ECM1	209365_s_at	0.7155617
LRRC15	213909_at	0.7155617
MMP11	203876_s_at	0.7155617
COPZ2	219561_at	0.71121013
PCOLCE	202465_at	0.6899231
THBS1	201108_s_at	0.68605226
GLT8D2	221447_s_at	0.68131775
-	221019_s_at	0.68131775
MMP19	204575_s_at	0.67816544
COL6A1	212091_s_at	0.6591419
COL6A2	209156_s_at	0.6591419
ANGPTL2	213004_at	0.6519295

ITGA5	201389_at	0.6519295
LOXL2	202998_s_at	0.6519295
RAB31	217762_s_at	0.6445346
PLAU	205479_s_at	0.6445346
VCAM1	203868_s_at	0.6445346
LPPR4	213496_at	0.6307415
ETV1	221911_at	0.6307415
COL16A1	204345_at	0.6217892
BGN	213905_x_at	0.6217892
C1QTNF3	220988_s_at	0.6091054
TIMP3	201147_s_at	0.6091054
-	214927_at	0.6091054
ITGBL1	205422_s_at	0.6091054
EPYC	206439_at	0.6091054
PRRX1	205991_s_at	0.60363436
EDNRA	216235_s_at	0.60363436

Table 9: Gene co-expression with INHBA in TCGA ovarian cancer samples (highlighted probes common with Table 4)

Gene Symbol	Reporter ID	Correlation
IL6	205207_at	1
HBEGF	203821_at	0.533625
CH25H	206932_at	0.492976
THBD	203887_s_at	0.492976
NR4A3	209959_at	0.492976
GEM	204472_at	0.492976
RGS2	202388_at	0.492976
SLC2A14	216236_s_at	0.391042
SLC2A3	202497_x_at	0.391042
SGK1	201739_at	0.334213
PTGS2	204748_at	0.304811
NR4A2	204621_s_at	0.293695
DUSP2	204794_at	0.293695
BTG2	201236_s_at	0.293695
ZEB1	208078_s_at	0.293695
RHOB	212099_at	0.293695
JUN	201464_x_at	0.293695
IER2	202081_at	0.293695
JUNB	201473_at	0.293695
KLF6	208961_s_at	0.293695
GADD45B	207574_s_at	0.293695

PPP1R15A	37028_at	0.293695
EGR2	205249_at	0.293695
CYR61	201289_at	0.293695
CTGF	209101_at	0.293695
ATF3	202672_s_at	0.293695
DUSP1	201044_x_at	0.293695
EGR3	206115_at	0.293695
NR4A1	202340_x_at	0.293695
FOSB	202768_at	0.293695
ZFP36	201531_at	0.293695
EGR1	201694_s_at	0.293695
FOS	209189_at	0.293695
CEBPD	203973_s_at	0.293695
EDN1	218995_s_at	0.293695
C10orf10	209182_s_at	0.293695
GADD45A	203725_at	0.293695
KLF10	202393_s_at	0.293695
BHLHE40	201169_s_at	0.293695
PLK3	204958_at	0.293695
DUSP5	209457_at	0.293695
LIF	205266_at	0.293695
MAFF	205193_at	0.293695
IER3	201631_s_at	0.293695

C8orf4	218541_s_at	0.293695

Table 10: Gene co-expression with IL6 in TCGA ovarian cancer samples (highlighted genes common with Table 4)

In vitro, treatment of Skov3-ip1 cancer cells with NE significantly induced *INHBA* expression, and pretreatment of HeyA8 cells with propranolol or ADRB2-antagonist butoxamine abrogated this effect (Figures 29a and 29b). Silencing ADRB2 using siRNA decreased *INHBA* expression in Skov3 cells after NE treatment (Figure 29c). NE can activate several transcription factors but primarily CREB1, Nf-kB and AP-1. Since Inhibin, Beta A gene was induced 3 hours after NE treatment I decided to start by looking at transcriptional activation of INHBA. TRANSFAC is a curated database for eukaryotic transcription factors, which provides promoter region and binding sequences for all genes. Using this, I identified CREB as potential transcription factor driving *INHBA* expression. Using TRANSFAC, I identified and designed primers specific to the promoter regions of INHBA where CREB1 binds. Chromatin immunoprecipitation analysis showed that NE increased CREB binding to the *INHBA* promoter 4-fold compared to normal IgG controls (Figure 29d). Silencing *CREB1* using siRNA also reduced NE-induced *INHBA* expression (Figure 29e). Conditioned medium from *CREB1*-silenced Skov3-ip1 cells also failed to increase ACTA2 expression in NOF151 cells, demonstrating the importance of Inhibin, Beta A in driving the NE-mediated CAF phenotype (Figure 29f).

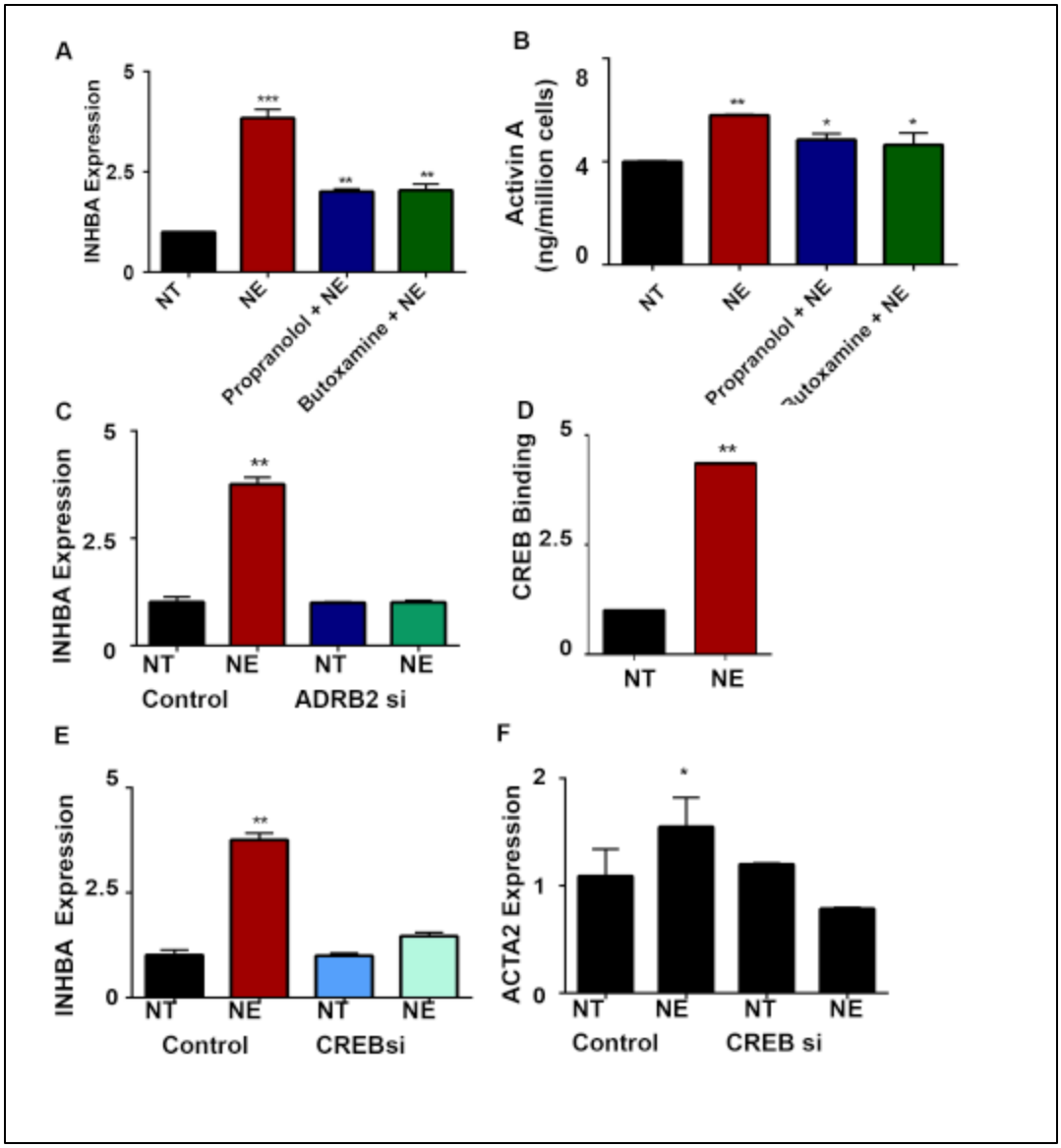


Figure 29: INHBA expression in tumor cells is mediated by ADRB2 and CREB

(a) Expression of *INHBA* in Skov3-ip1 cells after no treatment (NT) or treatment with norepinephrine (NE) with or without propranolol or ADRB2-specific blocker butoxamine. (b) Concentration of Inhibin, Beta A in HeyA8 cells after no treatment or treatment with NE with or without propranolol, or butoxamine. (c) Effect of silencing ADRB2 on *INHBA* expression in Skov3 cells not treated or treated with NE. (d) Chromatin immunoprecipitation analysis for CREB binding to

the *INHBA* promoter in Skov3-ip1 cells not treated or treated with NE. (e) Effect of silencing CREB on *INHBA* expression in Skov3 cells not treated or treated with NE.(f) Effect of medium conditioned by CREB1 siRNA– or control siRNA–treated cells not treated or treated with NE on CAF marker ACTA2 expression in NOF151 cells Scale bars, 100µM. Statistical significance was obtained using the 1-way ANOVA: *p < 0.05, **p < 0.01, ***p<0.001.

I then wanted to check if silencing INHBA in cancer cells also prevent induction of CAF-phenotype. I used several sequences for INHBA and validated knockdown using ELISA and qRT-PCR. Conditioned medium from *INHBA*-silenced cancer cells failed to increase ACTA2 levels in NOF151 (Figure 30a-30b). Functionally, NOF151 fibroblasts exposed to medium conditioned by INHBA-silenced Skov3-ip1 cells also showed decreased levels of the same collagens that were elevated in the patient tumor samples (Figure 30c and 30d). Taken together, these results indicate that NE drives the CAF phenotype *via* an ADRB2-CREB-INHBA axis.

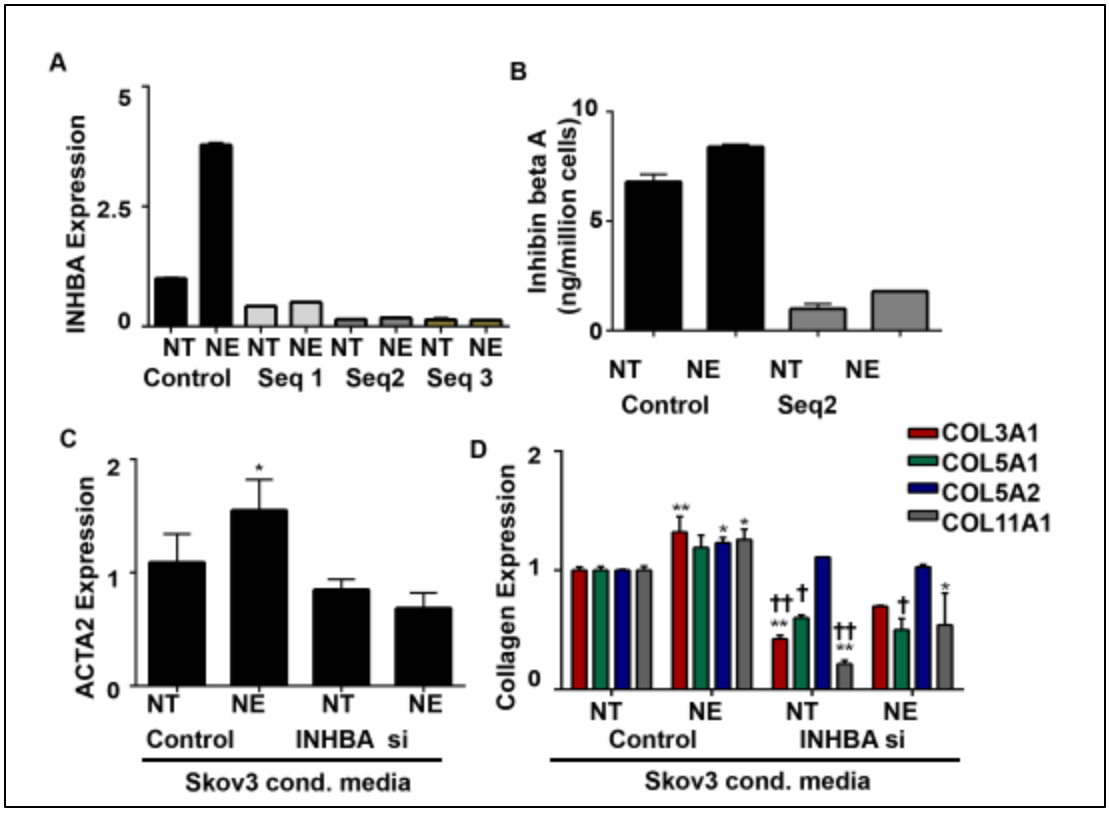


Figure 30: Conditioned media from INHBA-silenced tumor cells decrease CAF-phenotype and collagens in NOF151

(a) Validation of multiple sequences of *INHBA* siRNA in HeyA8 cells not treated (NT) or treated with norepinephrine (NE). (b) Concentration of Inhibin, Beta A in HeyA8 cells after *INHBA* was silenced via siRNA sequence 2 and no treatment or treatment with NE. (c) Effect of conditioned medium from INHBA siRNA– or control siRNA–treated Skov3 cells on expression of CAF marker ACTA2 in untreated or NE-treated NOF151 cells. (d) Effect of conditioned medium from ADRB2 siRNA– or INHBA siRNA– NE-treated Skov3 cells on collagen expression in NOF151 cells. Statistical significance was obtained using the 1-

way ANOVA: *p < 0.05, **p < 0.01 compared to control, †p<0.05 , ††p<0.01 compared to NE-treated cells.

I then wanted to silence INHBA in vivo to study stromal changes more directly in preclinical models. I used a nanoliposomal (1,2-dioleoyl-sn-glycero-3-phosphatidylcholine [DOPC]) system to silence INHBA in the tumor cells in the HeyA8 model. DOPC-based liposomes have been used to deliver siRNA and microRNAs to tumor cells efficiently in multiple models of cancer. Animals are treated with siRNA twice a week by IP injection. Restraint stress was induced using the physical restraint system described in the methods. Silencing INHBA decreased both tumor growth and metastasis (Figure 31a). Knockdown of *INHBA* was validated by both RNA expression using human-specific sequences (Figure 31b). More importantly, silencing Inhibin, Beta A decreased restraint stress–mediated increases in the CAF phenotype at both protein and mRNA levels (Figure 31c and 31d). Silencing INHBA in HeyA8 tumor cells decreased collagen deposition *in vivo* and also reduced levels of the collagens that were significantly elevated in the patient tumor samples (Figure 31e and Figure 31f).

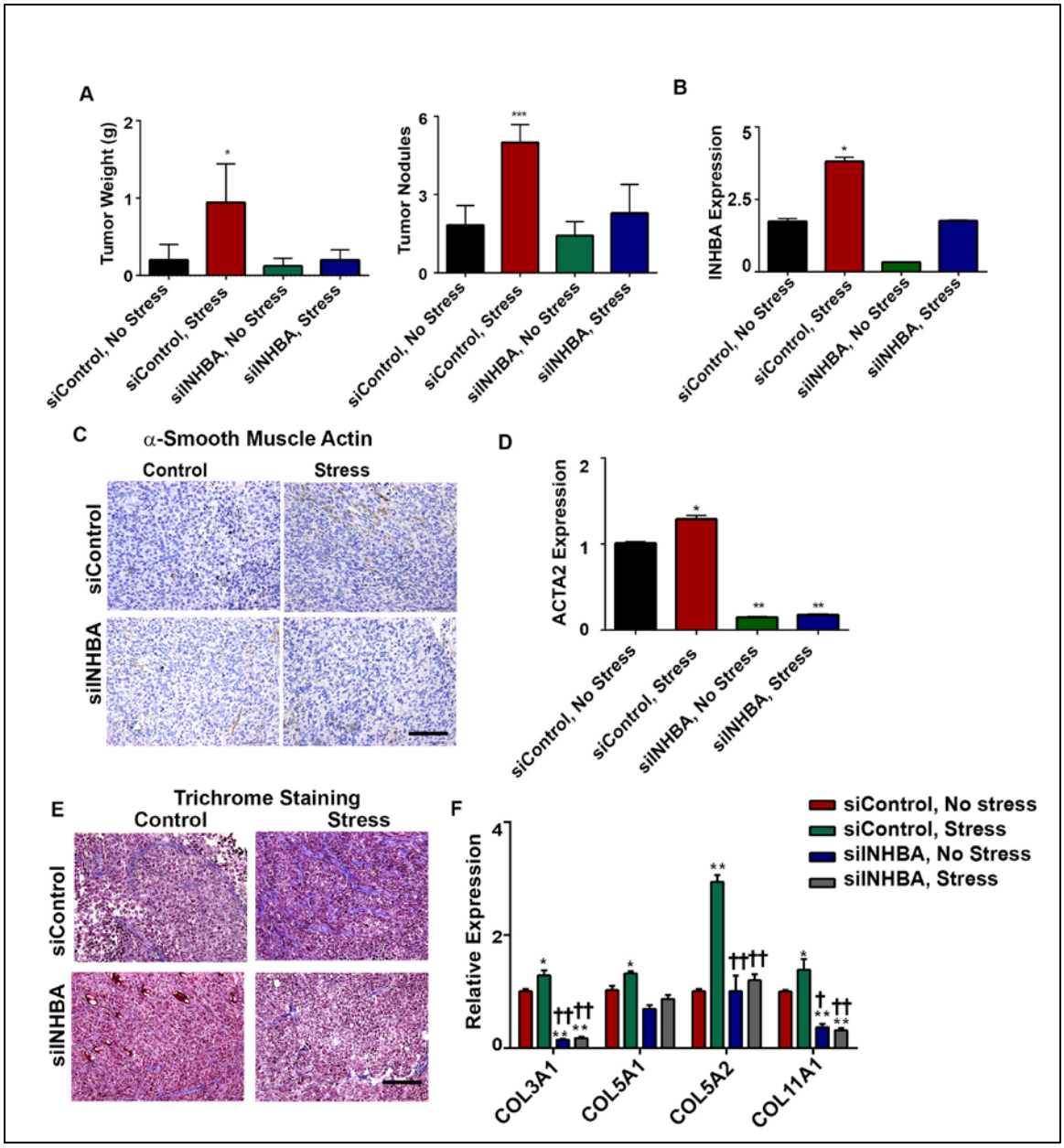


Figure 31: Effects of silencing INHBA in cancer cells

(a) Effects of silencing *INHBA in vivo* on tumor weight and tumor in nodules in orthotopic HeyA8 tumor-bearing mice subjected or not subjected (control) to daily restraint stress and treated twice per week with either control siRNA or *INHBA* siRNA. (b) Validation of knockdown of *INHBA* by qRT-PCR. (c) Expression of

CAF marker alpha–smooth muscle actin (α-SMA) in micrographs of representative HeyA8 tumors from control and stressed mice treated with control or INHBA siRNA (d) Expression of ACTA2 after INHBA silencing. (e) Expression of collagen identified by Masson trichrome staining in micrographs of representative HeyA8 tumors from control and stressed mice treated with control or INHBA siRNA. (f) Expression of collagens after INHBA silencing. Scale bars, 100μM, n=5/group for all data. Statistical significance was obtained using the 1-way ANOVA: *p < 0.05, **p < 0.01 compared to siControl-no stress, †p<0.05, ††p<0.01 compared to siControl-Stress.

I then focused on downstream signaling in NOF151 cells. Inhibin, Beta A functions via ACVR2a or ACVR2b receptors and downstream activation of Smad2/3 proteins. I started by staining the preclinical samples for both the receptors. Tumor stroma was weakly positive for ACVR2a and positive for ACVR2b (figure 32a). In vitro, I used NOF151 to silence both these receptors and studied changes in CAF and collagen genes. Silencing ACVR2b, not ACVR2a in NOF151 cells; abrogated changes in both ACTA2 and collagen expression mediated by NE-conditioned media (figure 32b). Further downstream of the receptors, SMAD proteins are activated by members of the TGFb pathway. Exposure of fibroblasts to media conditioned by NE-treated cancer cells resulted in a 3-fold increase in p-SMAD2 compared to media from non-treated cells (figure 32c).

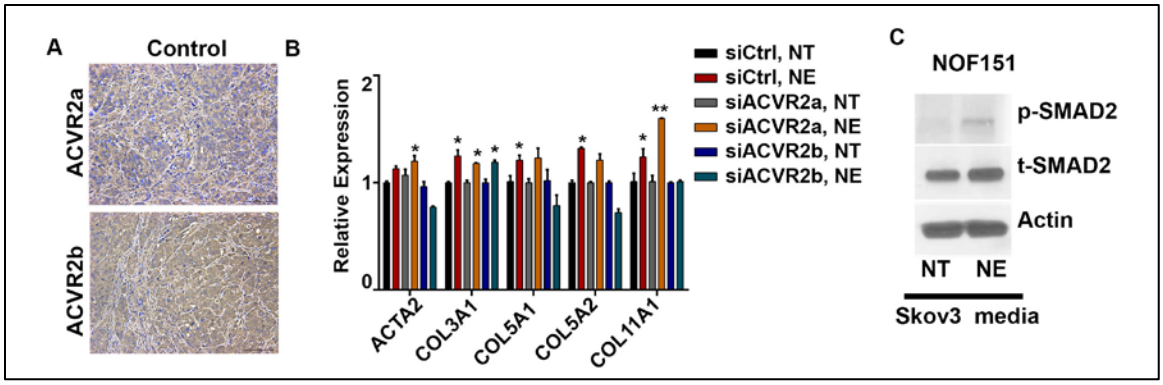


Figure 32: Role of Activin receptor in driving collagens

(a) Expression of ACVR2a and ACVR2b in ovarian tumor stroma. (d) Effect of NE-conditioned media after silencing ACVR2a and ACVR2b in NOF151 cells on ACTA2 and collagen expression. (c) Effect of NE-conditioned media on downstream SMAD2 activation in NOF151 cells. Statistical significance was obtained using the 1-way ANOVA: *$p < 0.05$, **$p < 0.01$ compared to siControl.

To check if *INHBA* affected survival outcomes, I analyzed the TCGA dataset using KMPlot. In 522 patients with high-grade serous ovarian cancer, primary tumors were evaluated for *INHBA* (210511_s_at) expression levels. All stages, TP53 status were included in the analysis with auto select of the best cutoff. Elevated *INHBA* expression was associated with significantly shorter overall survival in the TCGA ovarian cancer dataset (lower expression: 48.07 months, high expression: 39.57 months) (Figure 33a). Elevated *INHBA* expression was also associated with significantly progression-free survival in the TCGA ovarian cancer dataset (lower expression: 20.63 months, high expression: 15.63 months) (Figure 33a). Using Oncomine, I also assessed stage-wise expression of *INHBA* in ovarian cancer and there was an increase in expression associated with advanced stage (figure 33b).

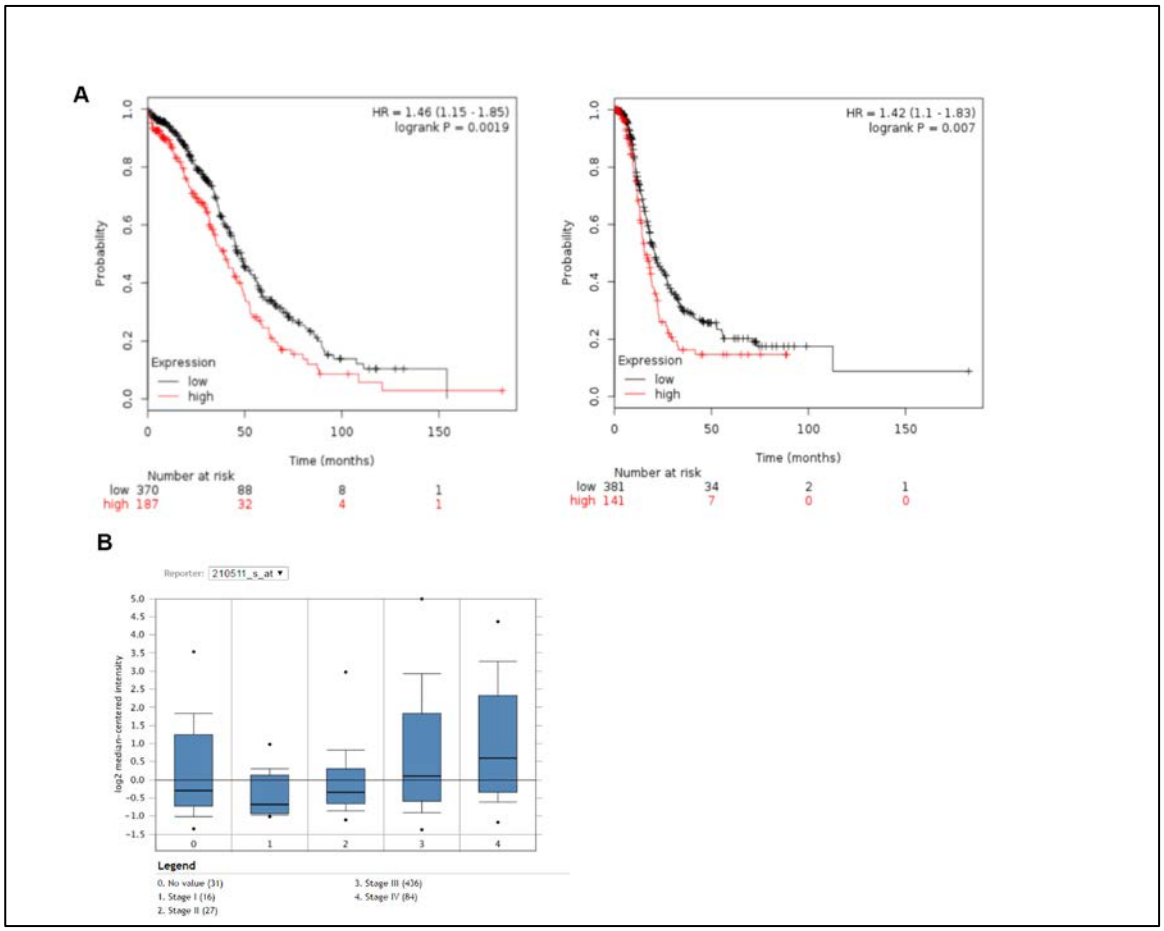


Figure 33: INHBA in ovarian cancer is associated with worse PFS and OS.

(a) Kaplan-Meier plots for overall survival (left) and progression-free survival (right) in patients with ovarian cancer, based on expression level of *INHBA*. Data were extracted from The Cancer Genome Atlas database. (b) Expression of *INHBA* with stage (TCGA data).

4.5 Adrenergic signaling–mediated CAFs modulate collagen in breast and colorectal cancers

Clinical and preclinical data have shown that chronic stress can exacerbate colorectal, breast, and prostate cancers in addition to ovarian cancer [88]. I wanted to study which other tumor types also show induction of the CAF phenotype during chronic adrenergic signaling. In both colon and breast cancers greater numbers of CAFs are associated with shorter survival, increased metastasis, and therapy resistance [130, 134, 162]. To determine whether our findings apply to other cancer types, we examined both clinical data and *in vivo* tumor samples from breast and colon cancer models. In mouse models generated from ADRB-positive RKO colon cancer cells or GILM2 breast cancer cells, α-SMA levels were significantly increased by restraint stress (2.2- and 3.4-fold increases for RKO and GILM2, respectively; $p<0.05$) (Figure 34a). There were concomitant increases in collagen in the tumors of mice subjected to restraint stress (1.7- and 2.1-fold increases for RKO and GILM2, respectively; $p<0.05$) (Figure 34b). Expression data from TCGA showed that INHBA levels are higher in tumors than in normal tissue (Figure 34c). Most intriguingly, analysis of genes that were co-expressed with *INHBA* in TCGA colorectal and ovarian cancers revealed significant overlap with the prominent CAF phenotype observed in ovarian cancer, and collagen patterns in these tumors were comparable to those in ovarian cancer (Tables 11 and 12). To further show the importance of chronic adrenergic signaling in promoting ECM- and CAF-related genes, we analyzed an independent set of renal carcinoma samples from patients with

known CES-D scores for collagens and CAF markers; the results show there is higher levels of gene expression for collagens, *INHBA* and *ACTA2* in patients with higher depressive symptoms (Figure 34d).

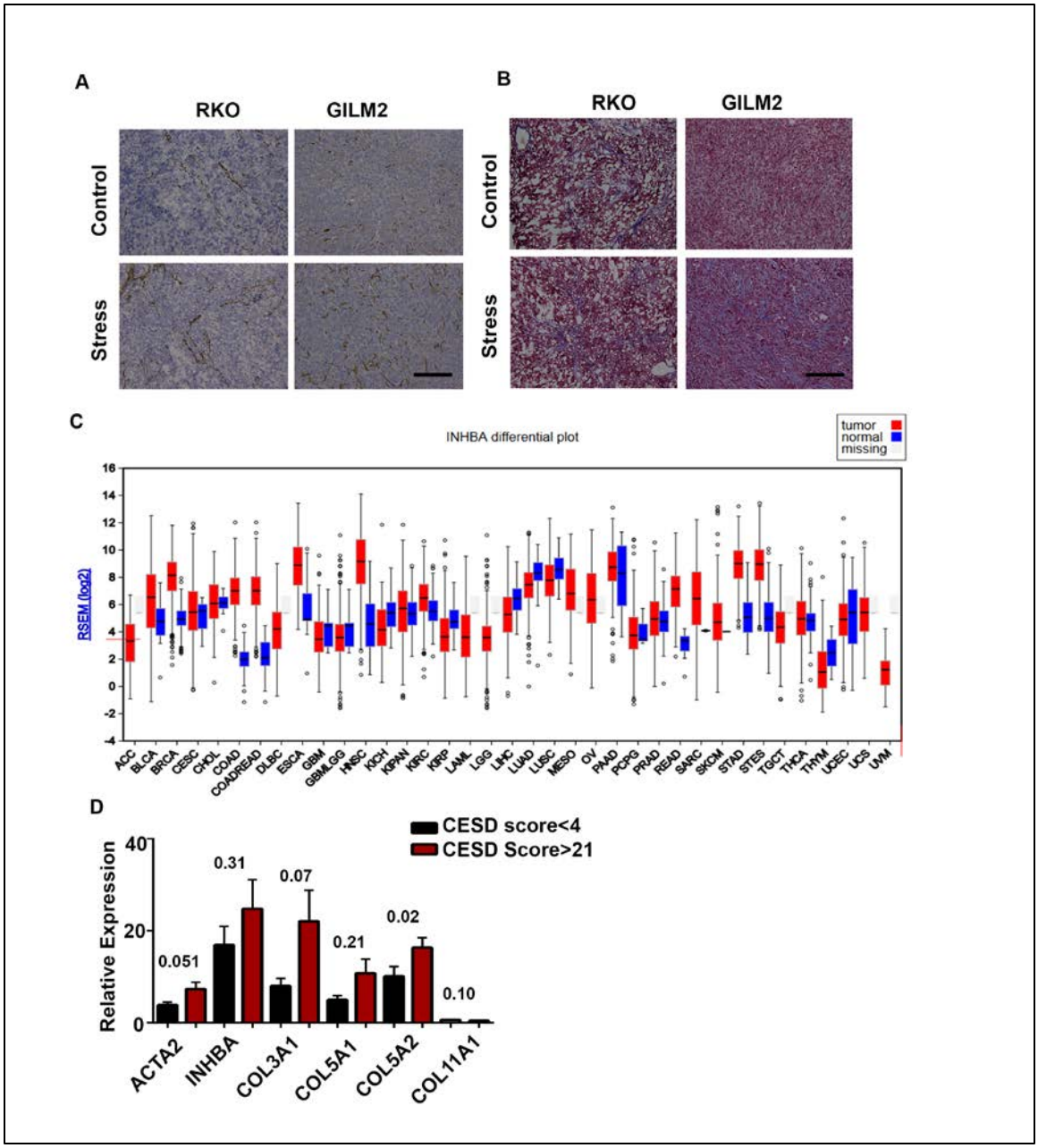


Figure 34: Restraint stress induces cancer-associated fibroblasts (CAFs) in colon, breast, and renal cancer models.

(a) Expression of CAF marker alpha–smooth muscle actin (α-SMA) in micrographs of representative tumors from control and restraint-stressed mice in adrenergic-receptor positive RKO (colon cancer) and GILM2 (breast cancer)

models. (b) Expression of collagen identified by Masson trichrome staining in micrographs of representative RKO and GILM2 tumors from control and stressed mice. (C) Pan-cancer INHBA expression from TCGA data. d)) Clinical validation of adrenergic-mediated CAF-phenotype in renal cell carcinoma samples using qRT-PCR p-values as indicated (total samples=9, 4 in CESD<4 and 5 in CESD>21). Scale bars, 100μM, Means ± SEM, n=5/group for RKO data and n=3/group for GILM2 data.

Breast Cancer		
INHBA	A_23_P122922	1
PPAPDC1A	A_24_P810284	0.847718
COL11A1	A_23_P11806	0.847718
KIF26B	A_23_P63541	0.804722
MMP11	A_23_P57417	0.79355
COL10A1	A_23_P214140	0.79355
GJB2	A_23_P407042	0.77325
FN1	A_24_P85539	0.756792
MMP13	A_23_P138931	0.713044
HSD17B6	A_23_P25030	0.704811
SPOCK1	A_24_P354689	0.675556
COL12A1	A_24_P291814	0.675556
CTHRC1	A_23_P111886	0.675556
COL3A1	A_24_P935491	0.675556
ASPN	NM_017680_2_2198	0.675556
AEBP1	A_23_P145918	0.675556
NOX4	A_23_P47147	0.675556
COL8A1	A_23_P69030	0.675556
COL1A2	A_24_P265274	0.675556
LOC651721	A_24_P282266	0.675556
WISP1	NKI_NM_003882	0.675556
LOC100128844	A_32_P141365	0.675556

P4HA3	A_23_P127956	0.675556
TMEM90B	NM_024893_1_1555	0.675556
LRRC15	A_24_P827032	0.675556
CDH11	A_23_P152305	0.675556
POSTN	A_24_P347411	0.675556
ADAM12	A_23_P202327	0.675556
COL6A3	NM_004369_1_9684	0.675556
VCAN	A_23_P144959	0.675556
COL1A1	A_23_P207521	0.675556
COL5A2	A_23_P10391	0.675556
COL5A1	A_23_P158590	0.675556
THBS2	A_23_P253652	0.675556
FAP	A_23_P56746	0.675556
TNFSF4	A_23_P126836	0.655745
LOC401097	A_23_P305243	0.655745
CILP2	A_23_P108238	0.642901
CORIN	A_23_P81131	0.634304
TLL2	A_23_P404778	0.610464
ERMN	A_23_P102017	0.609356
PLAU	A_23_P24103	0.609356
SULF1	A_23_P43165	0.609356
KIAA1199	A_23_P324754	0.609356
GREM1	A_23_P432945	0.571732

COMP	A_23_P90436	0.565048
PPEF1	A_23_P125505	0.543588
GRM8	A_32_P8221	0.543588
MATN3	NM_002381_2_2496	0.527919
C20orf103	A_23_P40294	0.527919
GRP	A_23_P101134	0.527919
ST6GAL2	A_32_P126157	0.527919
RGS4	A_23_P200737	0.510739
LOC285548	A_24_P892494	0.50405
NKX3-2	A_23_P386254	0.50405

Table 11: Gene co-expression with INHBA in TCGA breastl cancer samples (highlighted genes common with ovarian cancer)

Colorectal Cancer		
INHBA	A_23_P122922	1
COL11A1	NM_080629_1_6174	0.889562
ADAM12	NM_003474_2_4854	0.826843
NOX4	A_23_P47148	0.826843
CTHRC1	A_23_P111886	0.826843
FAP	A_23_P56746	0.826843
COL10A1	A_23_P214140	0.826843
COL5A2	A_32_P218731	0.811893
WISP1	NKI_NM_003882	0.809216

PDPN	A_23_P201322	0.809216
COL8A1	A_23_P69030	0.786018
ITGA11	A_23_P206022	0.786018
P4HA3	A_23_P127956	0.786018
SULF1	A_23_P43165	0.786018
THBS2	A_23_P253651	0.786018
PPAPDC1A	A_24_P810284	0.786018
COL1A1	A_23_P207521	0.786018
COL12A1	A_24_P291810	0.786018
COL6A3	NM_004369_1_9860	0.786018
COL1A2	A_23_P255244	0.786018
SPARC	A_23_P7642	0.786018
ZNF469	A_23_P335080	0.786018
CDH11	A_23_P152305	0.786018
KAL1	A_23_P429948	0.754175
LOC651721	A_24_P282266	0.744667
HS3ST3A1	A_23_P66523	0.72371
POSTN	A_23_P205111	0.72371
LOX	NM_002317_3_1867	0.72371
TWIST1	A_23_P71067	0.715534
ST6GALNAC5	A_23_P33093	0.698786
LUM	A_32_P28664	0.698786
TMEM90B	A_24_P190504	0.698786

FBN1	A_24_P152553	0.698786
ANTXR1	A_24_P131522	0.698786
NTM	A_23_P84060	0.698786
SPOCK1	A_23_P81598	0.698786
HTRA3	A_23_P395438	0.698786
TNFSF4	A_23_P126833	0.672474
MXRA5	A_24_P282355	0.662766
ADAMTS6	A_23_P213319	0.632247
KIF26B	A_23_P63541	0.632247
CNIH3	A_23_P384044	0.625946
SHISA2	A_32_P55236	0.625946
MMP11	A_23_P57417	0.625946
PPEF1	A_23_P125503	0.625946
MFAP2	A_23_P1027	0.625946
LRRC15	A_24_P827037	0.616798
HMCN1	A_23_P148991	0.616798
CRISPLD1	A_23_P59958	0.616798
COMP	A_23_P90430	0.616798
ITGBL1	A_23_P113777	0.616798
SFRP4	A_23_P215320	0.616798
KCNE4	A_23_P392574	0.604307
DIO2	A_23_P48736	0.581334
CLEC5A	A_23_P304356	0.550313

SLN	A_23_P150343	0.550313
C5orf46	A_23_P19176	0.550313
FGF1	A_23_P213330	0.550313
OLR1	A_24_P124624	0.550313
SPP1	A_23_P7311	0.550313
MMP13	A_23_P138931	0.54776
ALPK2	A_23_P15876	0.54776
PRRX1	A_23_P502731	0.54776
FN1	NM_002026_1_7942	0.541192
DKK2	A_23_P155847	0.52731
GRP	A_23_P101134	0.52731
EDNRA	A_24_P217572	0.521915

Table 12: Gene co-expression with INHBA in TCGA colorectal cancer samples (highlighted genes common with ovarian cancer)

5 Summary and Future Directions

5.1 Summary

A growing number of studies have shown a role of elevated adrenergic signaling in driving tumor progression and adversely affecting survival. Epidemiological studies, preclinical and *in vitro* studies in ovarian, lung, prostate, breast, renal and colon cancers have shown norepinephrine can activate pro-survival and metastatic networks on cancer cells that result in increased tumor growth and decreased survival [88].

All the studies studying adrenergic influences focused on a particular aspect of tumor progression, but in this study we start with an unbiased analysis to identify novel pathways that are influenced by norepinephrine. Using clinical data from patients with known biobehavioral profiles it enabled us to identify pathways that included both cancer cells and stromal components.

Tumor microenvironment is a complex milieu with multiple host cells, including macrophages, endothelial cells, infiltrating immune cells, and cancer associated fibroblasts. Data showing effects on stroma is limited and was pursued further in this study. In this dissertation, I focused on the effect and mechanism by which catecholamine signaling in drives stromal changes in the microenvironment in ovarian cancer (figure 35). Using gene expression data patients with known depressive scores, I first identified a prominent association between depressive symptoms and CAF phenotype. Cancer associated fibroblasts are important in ovarian cancer, and have functions in driving metastasis and immunosuppression [133]. Therefore studying the role of chronic stress in driving CAFs in tumors can further explain some of the documented effects on tumor

progression. Several genes that were elevated such as *THBS2, ACTA2,* and *SPARC* are also established CAF markers. Elevated gene networks in patients with high depressive symptoms include those associated with proteinaceous extracellular matrix, collagens, and epidermis development primarily various collagens. I then validated this CAF phenotype using both *in vitro* and *in vivo* studies. Staining for a-SMA which is a prominent CAF marker, there are significantly increased levels in Skov3, HeyA8 and ID8-VEGF *in vivo* models. *In vitro,* normal ovarian fibroblasts that were exposed to NE-conditioned media from cancer cells showed accelerated CAF induction. To identify the mechanism leading to CAF-phenotype, I utilized both bioinformatic and molecular biology approaches, and identified a novel mechanism of fibroblast activation triggered by Inhibin, Beta A production by tumor cells in response to sustained adrenergic stimulation. Inhibin, Beta A is a member of the TGF-b pathway that has direct role on cancer cells in promoting tumor growth, but no data exists showing a role in CAF phenotype. In TCGA clinical samples, *INHBA* was also highly correlated with genes that were identified to be elevated in patients with high depressive scores. Inhibin, Beta A production by cancer cells after NE treatment was triggered by an ADRB2-CREB axis and silencing ADRB2 or CREB using siRNA abrogated NE-induced Inhibin, Beta A production. Additionally, fibroblasts exposed to conditioned media treated with from ADRB2 and CREB silenced cells also did not show increases in CAF genes such as *ACTA2, S100A4* and *FAP*.

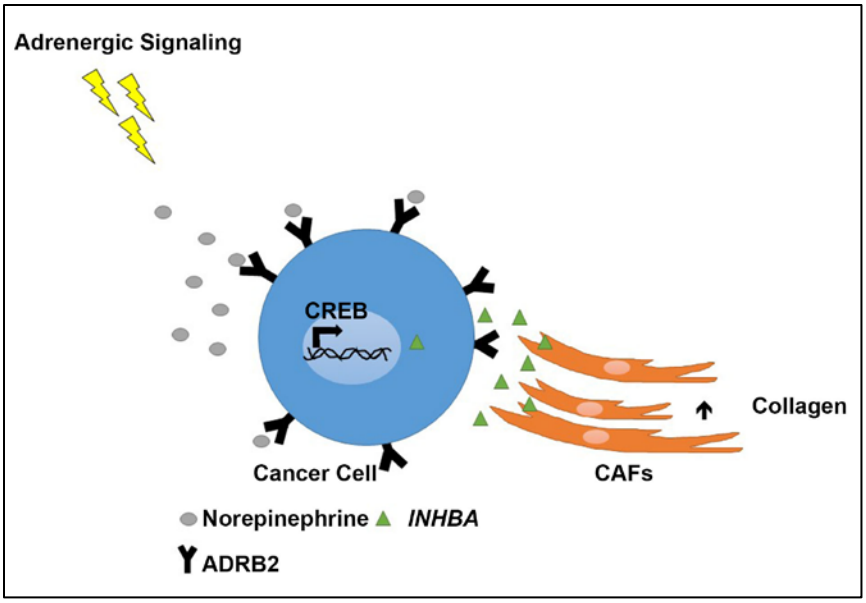


Figure 35: Summary

To determine the biological consequences of adrenergic-mediated CAF phenotype, I focused on the extracellular matrix and collagen proteins. Using histological Trichrome staining, I identified levels of several collagens such as *COL3A1, COL5A1, COL5A2* and *COL11A1* are driven by downstream Inhibin, Beta A-signaling in CAFs and were elevated during adrenergic signaling. When animals were treated with beta-blockers, stress-related collagen increases in tissue was eliminated. I also silenced Inhibin, Beta A in cancer cells using liposomal nanoparticles containing siRNA, and this abrogated all collagen and CAF changes in tumors.

To extend our findings to other cancers, we also studied colon and breast cancer models using RKO and GILM2 *in vivo* models respectively. Chronic stress significantly increased a-SMA, Inhibin, Beta A and collagen levels similar to ovarian cancer. In addition, in both cases *INHBA* was also associated with high

co-expression of collagen and CAF genes. Using an independent dataset of renal cell carcinoma patients with known depression scores, there was a trend towards increased *ACTA2, INHBA* and collagen genes associated with increased CESD scores.

Work done in this project extends our understanding on how catecholamines can affect the tumor microenvironment. For the first time, we have shown that *INHBA* can be induced by NE stimulation in cancer cells, and this can induce a prominent CAF phenotype in ovarian cancer. Association studies have shown *INHBA* closely tracks with collagen genes in cancer, and we found similar results using TCGA in ovarian, colon and breast cancers. However, our studies differ from those published in that we show the association is casual and is driven by INHBA inducing collagen genes. We provide evidence the silencing INHBA can have profound effects on tumor stroma especially CAFs.

Although the principal effect is still on tumor stroma, the effect is still mediated by norepinephrine acting on cancer cells. Blocking ADRB signaling in tumor cells caused decreases in stromal content and CAF phenotype in several cancer types. With increasing number of studies that are focusing on the role of chronic stress in exacerbating diseases including cancer, we provide another motivation to combine beta blockers with conventional therapy. Beta blockers are approved by the FDA for heart disease (and infantile hemangioma) and are in clinical trials for ovarian cancer. Propranolol is well tolerated and has very few side effects, and provides another very targetable drug to block stromal changes.

5.2 Future Directions

Chronic stress and NE-mediated ADRB2 and CREB signaling in cancer cells is important for increasing angiogenesis, tumor invasion and inflammation. In cardiac tissues, NE can promote fibrosis by increasing proliferation of primary cardiac fibroblasts in a TGFb1-dependent pathway [163]. CREB driven Inhibin, Beta A signaling in hippocampus is neuroprotective by reducing excitotoxic neuronal damage [164]. Inhibin, Beta A is a member of the TGF-β pathway and is overexpressed in prostate, ovarian, colorectal, and breast cancers [151, 165, 166]. Elevated serum and plasma levels of Inhibin, Beta A have been reported in patients with metastatic ovarian, breast, and prostate cancers [151, 165, 166]. In our study, silencing Inhibin, Beta A in tumor cells reduced the levels of adrenergic stimulus induced CAFs and collagen significantly. Although co-expression of collagen genes with *INHBA* has been reported in some cancers, the functional relationships have not been well understood [167].

Several studies have identified the direct role of NE in driving molecular changes in tumor cells but influences on stroma have been limited to natural killer cells, macrophage infiltration and angiogenesis [88]. Effects of adrenergic signaling on CAFs and extracellular matrix provide a previously unrecognized dimension that could have therapeutic implications. CAFs are the major part of tumor stroma and provide tumor cells with vital cues for invasion by remodeling the microenvironment through synthesis and deposition of collagens and producing pro-inflammatory cytokines and other growth factors[133]. Targeting stroma can lead to several clinical benefits including increased immune cell infiltration and

decreased metastasis Collagens in the extracellular matrix can engage integrins on tumor cells, impede T-cell infiltration and facilitate invasion and metastasis. Importantly, collagens identified in our study (e.g., COL5A1*, COL11A1* and *COL1A1*) are also associated with increased metastasis and poor overall survival in ovarian cancer [168].

5.3 Study Impact

Cancer diagnosis and treatment are extremely stressful events for patients and families, and American Psychological Association also frequency reports that chronic stress events in individuals are on the rise. Hence, studying the interplay between stress hormones and their effects on tumor biology is becoming increasingly important. To our knowledge, this is the first report that studies adrenergic influences on tumor growth in a completely unbiased manner. Although the role of chronic stress and psychological parameters are shown to exacerbate cancer have been shown in several cancers, thoughtful pharmacological and biobehavioral interventions to prevent this are few. Biobehavorial interventions for cancer patients has been studied since 1970. Some of the interventions that have been tried and proven to yield success include regular therapy, relaxation training, social adjustment and also health behaviour such as exercise, dietary modifications and strategies for symptoms management (e.g., nausea, sleep deprivation). Biobehavioral interventions have several limitations, including but not limited to high costs, treatment dropout, and lower adherence to interventions. Hence, beta-blockers which are FDA-approved with well tolerated side effects would be the ideal to treat chronic stress in cancer

patients. In infantile hemangioma, beta blockers are the standard of care with Beta blockers are currently being tested in clinical trials in combination with chemotherapy in ovarian cancer. Understanding the various modes by which catecholamines will affect tumor microenvironment will enable us to design novel drug combinations with beta blockers. Emerging data from molecular underpinnings that drive NE-induced tumor growth can be harnessed further to effectively combine therapeutic interventions (such as beta blockers) and behavioural interventions with novel antagonists and inhibitors. There is data to show that beta blockers can combine well with NSAIDs such as etodolac and aspirin by blocking direct effects of NE and downstream inflammation signaling. As studies continue to support the many molecular mechanisms that drive NE-mediated tumor growth, several targeted therapies (such as bevacizumab or immunotherapy) can be combined in large scale randomized studies to study clinical benefits.

Inhibin, Beta A was identified in the study to be the most important extracellular factor produced by cancer cells that can drive CAF phenotype in tumors. To our knowledge, this is the first report to show that Inhibin, Beta A is necessary for increased collagen levels in CAFs and may be an important biomarker for tumor progression. This was not limited to ovarian cancer, but similar changes in NE-induced Inhibin, Beta A increased collagen levels in breast and colorectal preclinical models. High depressive scores was also associated with high expression of Inhibin, Beta A and collagens in renal cell carcinoma. Several studies have focused on targeting Inhibin, Beta A to prevent cancer cachexia, but

our results demonstrate important roles in modulating the tumor stroma [169-171] that can be targeted to prevent potential tumor metastatic and increase T-cell infiltration.

STM434, a humanized decoy receptor for activin receptor, is currently in a clinical trial for treating patients with metastatic and chemotherapy resistant ovarian cancer (NCT02262455). Single agent STM 434 showed an acceptable safety profile in patients with advanced solid tumors and early evidence of clinical activity. Beta-blockers are associated with survival benefit in several cancers and our results suggest combining beta-blockers with novel targeting agents such as Inhibin, Beta A decoy receptors in treatment regimens. Disruption of interactions between cancer cells and stress hormones or cancer cells and fibroblasts may improve outcomes of cancer patients. As the importance of Inhibin, Beta A and collagens in driving cancers emerge, several therapies targeting them can be combined with beta-blockers to improve patient survival in ovarian, colon, breast and renal cell cancers.

6 References

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7 Vita

Archana Sidalaghatta Nagaraja was born March 13, 1985 in Bangalore, India. She is the younger of 2 children of Sukanya T. and Nagaraja S. R., with older brother Ajay. After graduating from Vijaya PU College, Bangalore, India in 2003 she attended the RV College of Engineering, Bangalore, India. In May 2007 she graduated 4th in class from Visvesvaraya Technological University with a Bachelor's of Engineering in Biotechnology. She then worked at Cerner Healthcare Systems in Bangalore, India. She then entered Drexel University in September 2008 and joined the laboratory of Dr. Margaret Wheatley in January 2009. She graduated in September 2010 with her Masters of Science in Biomedical Engineering with her thesis on 'Ultrasound Contrast Agents to deliver curcumin to tumor cells'. She then worked at Norris Cotton Cancer Center at Dartmouth College in Dr. Alexey Danilov's lab (Dr. Murray Korc). She then entered the The University of Texas MD Anderson Cancer Center UT Health Graduate School of Biomedical Sciences in August 2011 and joined the laboratory of Dr. Anil Sood in March 2012.